

**THE LENTIL-MEAT SYSTEM: INVESTIGATING THE ANTIOXIDANT EFFECT OF
LENTIL ON COLOUR AND LIPID OXIDATION OF RAW BEEF BURGERS**

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By
He Li
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ABSTRACT

Discoloration and lipid oxidation are the main deteriorative causes of raw meat products. The flour of lentil, when heat treated, was found to protect fresh meat colour and inhibit lipid oxidation when incorporated into raw meat products. In order to postulate a possible mechanism of this useful functionality of lentil flour, three studies were conducted. In the first study, the impact of infrared heating to 115 and 150 °C and water bath heating of 90 °C (30 min) of different seed components of two Canadian lentil cultivars were evaluated. Enzyme activities, soluble proteins and phenolics that promote and negate oxidation reactions were assayed. The second study was designed to investigate the effects of seed coat and cotyledon with or without heat treatment in relation to enzyme and antioxidant activities in the ground meat system. It was investigated the effects on colour parameters (L^* , a^* and b^*) and myoglobin redox states (met-, oxy- and deoxy-) of the product surface and lipid oxidation (thiobarbituric acid reactive substances: TBARS) during the refrigerated storage of lentil-ground meat product for 7 days. In the third study, the usability of lentil as a binder was evaluated when ground beef burgers containing the same levels of lentil components were stored for 12 weeks under frozen (-20 °C) condition, in terms of the effect on colour, myoglobin redox states and lipid oxidation.

In the first study, it was found that the lipoxxygenase, peroxidase and glutathione reductase activities were mostly found in cotyledon rather than in seed coat. The seed coat exhibited higher superoxide dismutase activity than cotyledon. The heat treatments tested were able to deactivate lipoxxygenase, peroxidase and glutathione reductase significantly ($P < 0.05$), but not the superoxide dismutase ($P > 0.05$). Heat treatments significantly ($P < 0.05$) increased Fe^{2+} chelating activity for all samples. Soluble proteins in the seed coat (hull) showed higher antiradical (1,1-diphenyl-2-picryl-hydrazyl: DDPH and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid): ABTS) and antioxidant (ferric reducing antioxidant potential: FRAP, Fe^{2+} chelating) activities than those obtained from the cotyledon, and this corresponded with the higher amount of protein-bound phenolic compounds. The extracts (water and 70% (v/v) ethanol) of the seed coat contained a

higher ($P<0.05$) level of total phenolics, condensed tannins and total flavonoids than cotyledon extracts. Water and 70% (v/v) ethanol extracts from the seed coat were able to reduce approximately 10 - 20% metmyoglobin to form oxymyoglobin, but the cotyledon extract could not. The addition of metmyoglobin was found to dramatically increase the rate of linoleic acid oxidation by 1000 fold. However, the addition of 70% (v/v) ethanol and water extracts of seed coat were able to significantly lower ($P<0.05$) such high oxidation rate initiated by metmyoglobin. The water extracts of cotyledon when used at higher levels were able to lower the oxidation rate, but were not as effective as the water extract of seed coat.

In the second study, it was found that the heat-treated lentil flour (whole seed) delayed the lowering of a^* value (redness) of beef burgers and reduced the lipid oxidation product generation ($P<0.05$) during the 7-day storage compared with the controls. However, the products with raw lentil flour (whole seed) accelerated the decrease in a^* value. It was also observed that the flour of seed coat and cotyledon separated from heat-treated seeds were able to delay discoloration of beef burgers ($P<0.05$), however, raw cotyledon flour could not. Burgers with added seed coat flour either from raw or heat-treated seeds developed lower ($P<0.05$) level of TBARS than control burgers and those with cotyledon flour at the end of storage. Negative correlations between redness and metmyoglobin and between redness and TBARS were obtained.

In the third study, no difference ($P>0.05$) was observed on the a^* value of the burgers containing heat-treated flour and raw flour that were stored for 12 weeks under $-20\text{ }^{\circ}\text{C}$ while all samples showed higher a^* values than the control. The burgers containing cotyledon flour of heat-treated seeds showed lower level ($P<0.05$) of metmyoglobin than the burgers with seed coat flour. Burgers containing all types of lentil flour had a lower ($P<0.05$) level of TBARS generated than the control group. Among these flour types, a lower level of lipid oxidation ($P<0.05$) occurred in the burgers containing heat-treated lentil flour than the ones containing raw flours. Within burgers with added raw seed flour, the addition of cotyledon one showed higher TBARS

values than those containing seed coat one ($P<0.05$). Significant negative correlations were found between a^* value and metmyoglobin and between a^* and TBARS values.

Overall, it was found that the antioxidant activity of the water soluble components of the lentil seed is the main factor that protects colour and retards lipid oxidation in raw meat products, via metmyoglobin reduction, Fe^{2+} chelating, free radicals scavenging and inhibition of unsaturated lipid oxidation catalyzed by metmyoglobin. The pro-oxidative activities of lentil components are mainly due to the oxidative enzymes and these enzymes are more sensitive to heat. The performance of lentil flour differs in meat products under refrigerated and frozen conditions. But heat-treated lentil flour can be considered more stable in providing colour protection and inhibiting lipid oxidation during storage.

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LIST OF ABBREVIATIONS

AACC	American association of cereal chemists
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ANOVA	Analysis of variance
AOAC	Association of official analytical chemists
CTC	Condensed tannin content
DeoxyMb	Deoxymyoglobin
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
FRAP	Ferric reducing antioxidant potential
GLM	General linear model
LDL	Low density lipoprotein
Mb	Myoglobin
MetMb	Metmyoglobin
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitro blue tetrazolium
OxyMb	Oxymyoglobin
ROS	Reactive oxygen species
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TFC	Total flavonoid content
TPC	Total phenolic content
TPTZ	2,4,6-tripyridyl- <i>s</i> -triazine

1 INTRODUCTION

Meat colour is a critical property of raw meat products. It is believed that the colour, rather than texture or flavour is the determining factor affecting consumers' purchase behavior of fresh meat (Carpenter, Cornforth and Whittier, 2001). However, fresh colour of meat tends to change during packaging, storing and retailing from its original bright red colour to dark brown colour, due to the oxidation of its major pigment, myoglobin (Mancini & Hunt, 2005). Over the years, many researchers conducted studies on slowing down the discoloration and myoglobin oxidation using various approaches, such as applying modified atmosphere packaging, using antioxidants either added in animal's diet or in ground meat products (Suman & Joseph, 2013; Faustman, Sun, Mancini, & Suman, 2010; McMillin, 2008). Antioxidants from natural sources such as seed extracts, leaf extracts and spice extracts have been widely studied for meat colour protection (Suman & Joseph, 2013).

Lentil is a widely produced pulse plant in Saskatchewan, Canada (Statistics Canada, 2012a). It was found that heat-treated lentil flour when incorporated into beef burgers protected raw meat colour under refrigerated storage condition while the raw lentil flour accelerated discoloration of beef burgers (Der, 2010). This suggests that the heat-treated lentil flour may pose a strong antioxidant effect on meat in protecting colour, while raw lentil flour poses an opposite effect.

It was also found that the raw lentil flour contains high level of lipoxygenase activity (Der, 2010; Pathiratne, 2014) and peroxidase activity (Pathiratne, 2014). These oxidative enzymes may accelerate lipid oxidation in the lentil and in meat products. The lipid oxidation products cause undesirable flavour alterations and elevate oxidative stress of the overall meat system. In addition, lipid oxidation and myoglobin oxidation are interrelated. The products of lipid oxidation

such as 4-hydroxy nonenal (4-HNE) are able to accelerate the oxidation of oxymyoglobin, consequently causing discoloration of meat products (O'Grady, Monahan, & Brunton, 2001). Although it can be postulated that raw lentil flour induces colour deterioration and accelerated lipid oxidation in beef burgers, little is known on the distribution of oxidative enzymes and antioxidants across seed physical components, namely the seed coat and cotyledon. Also, limited information is available on the effect of heat treatment on these lentil components and the possible mechanism of how these components react with myoglobin and lipids in a meat product.

Lentil seed contains high amounts of protein, starch and fibre, but is low in fat content (Candela, Astiasaran, & Bello, 1997). Unlike wheat, lentil is regarded as a gluten-free food grain (Shepherd & Gibson, 2006). This compositional feature makes lentil flour a potential binder for meat products with added useful traits. The incorporation of lentil flour into beef burgers resulted in improved cooking yield and water retention of low-fat beef burgers, and decreased the product shear force and hardness (Der, 2010). Also, the overall acceptability was not affected by addition of lentil flour in beef burgers (Der, 2010). These results suggest that lentil flour can be a valuable binder in meat products to enhance certain textural properties.

This background information led us to investigate the diametrically opposed effects of heat-treated and raw lentil flour on meat colour and lipid oxidation of raw meat products. Therefore, the objectives of this study were:

- 1) To determine various enzyme activities and antioxidant potential of soluble protein extracts of whole seed, cotyledon and seed coat of two lentil cultivars (representing two market classes) with or without heat treatment. Also, investigation of the metmyoglobin reducing ability and linoleic acid oxidation prevention of the ethanolic or water extracts of different lentil flour types using model systems was envisaged.

2) To investigate the effects of lentil flour obtained from different lentil types (cultivars and seed components) on colour, myoglobin oxidation and lipid oxidation of raw beef burgers during refrigerated storage.

3) To investigate the effects of lentil flour (similar to objective 2) on colour, myoglobin oxidation and lipid oxidation of raw beef burgers during frozen storage.

This thesis is presented in the manuscript style. The main chapters are as follows: Chapter 2 presents the literature review and three studies corresponding to the above mentioned three objectives are presented in Chapters 3, 4 and 5, respectively.

2 LITERATURE REVIEW

2.1 Lentil composition

Lentil (*Lens culinaris*) is an old crop which originated from Near East, in an area called “the cradle of agriculture” (Sonnante, Hammer, & Pignone, 2009). It has been an important food since prehistoric times and has been cultivated by human beings since 6800 BC (Yadav, McNeil, & Stevenson, 2007). In 2010, over 4 million hectares of lentil were cultivated all over the world, and the overall production of lentil seed in 2010 is over 4,600,000 tonnes (FAO, 2013). Canada is the largest producer of lentil in the world (FAO, 2013). The overall production of Canadian lentil in 2011 was over 1,500,000 tonnes while Saskatchewan contributes the largest portion, over 1,303,000 tonnes (Statistics Canada, 2012a).

Lentil is considered as a good source of protein. Total amount of protein in lentil seed accounts for over 25% of its dry weight. The lentil protein provides both essential and non-essential amino acids for metabolism by the human body (Faris, Takruri, & Issa, 2013). Lentil also contains high content of carbohydrates, which make up almost 60% of dry mass in lentil seed and makes it an excellent source of starch and total dietary fibers. The fat content in lentil seed, however, is relatively low, which contributes to its low energy content (Candela et al., 1997). All of these compositional characteristics make lentil a potential meat product binder which can be utilized further for the meat industry. Besides those macro components, there are phytochemicals present in lentil at lower level, but acting an important role. Antioxidants such as phenolic compounds, vitamin C and vitamin E are such phytochemicals contributing health benefits to human bodies and antioxidant effects to food matrices (Han & Baik, 2008).

2.1.1 Protein and amino acids

The major form of protein found in lentil seed is storage protein, a reservoir of various amino acids (Roy, Boye, & Simpson, 2010). Based on the solubility properties, Roy et al. (2010) and Boye, Zare and Pletch (2010) investigated lentil proteins according to Osborne classification as albumins, globulins, glutelins and prolamins. Albumins are the water soluble proteins and comprise enzymes, protease inhibitors, amylase inhibitors and lectins. Their molecular masses range from 5,000 to 80,000 Daltons (Roy et al., 2010). Globulins are the salt soluble proteins and the major globulins found in lentil are legumin (11S) and vicilin (7S). Legumins contain acidic subunits (~40,000 Da) and basic subunits (~20,000 Da), while vicilins have a molecular mass of 175,000 - 180,000 Daltons (Roy et al., 2010). Glutelins are soluble in dilute acids or alkaline detergents and prolamins are the alcohol soluble proteins (Boye et al., 2010). Also, lentil proteins do not contain gluten, therefore its flour can be considered as gluten-free and useful in gluten-free product development such as for baking gluten-free crackers, bread or other gluten-free food (Shepherd & Gibson, 2006).

The amino acid content of lentil is presented in Table 2.1 and it shows that the lentil proteins are generally high in glutamic acid, aspartic acid, arginine and leucine, but low in methionine, tryptophan and cysteine. The amino acid profile of lentil makes it a good supplement for cereal food, such as wheat, rice or corn that are low in lysine (Bhatty, 1988).

Table 2.1 Contents of amino acids in lentil seed

Type	Amino acid	Amount (g/100 g wet sample) ^a	Amount (g/100 g dry sample) ^b
Essential	Histidine	0.18	0.874
	Isoleucine	0.50	0.626
	Leucine	0.63	1.064
	Lysine	0.32	0.454
	Methionine	0.05	0.149
	Phenylalanine	0.26	0.670
	Threonine	--	0.557
	Tryptophan	--	--
	Valine	0.76	0.854
Non-essential	Alanine	0.61	2.042
	Arginine	--	1.061
	Aspartic acid	0.22	1.096
	Cysteine	--	0.040
	Glutamic acid	2.23	2.655
	Glycine	0.35	0.977
	Proline	0.37	1.111
	Serine	0.01	1.138
	Tyrosine	0.15	0.634

^a Candela et al. (1997)^b Rozan, Kuo, & Lambein (2001)

2.1.1.1 Enzymes

Most enzymes are proteins and in lentil seeds they offer different functions. Some enzymes catalyze redox reactions and could be oxidative or antioxidative. Lipoxygenase (linoleate 13S-lipoxygenase, EC 1.13.11.12) is an oxidative enzyme that catalyzes hydroperoxidation with one molecular oxygen of polyunsaturated fatty acids containing *cis,cis*-1,4-pentadiene, such as linoleic acid and linolenic acid (Khalyfa, Kermasha, & Alli, 1990). The lipoxygenase activity of lentil seed is found to be higher than other legumes, such as pea, chickpea and faba bean (Chang & McCurdy, 1985). Lipoxygenase in lentil seed may play a physiological role either during seed maturation or during germination and seedling growth (Porta & Rocha-Sosa, 2002). Although the

functions of lipoxygenase are not fully understood yet, it may function in fatty acid peroxidation in membranes or storage lipids, production of growth regulators as a response to pathogens and as nitrogen storage (Loiseau, Ly Vu, Macherel, & Deunff, 2001). Peroxidase (EC 1.11.1.7) is another oxidative enzyme that can catalyze the reduction of hydrogen peroxide by oxidizing substances such as phenolic compounds (Nakamoto & Machida, 1992). Activities of both these enzymes are believed to cause off-flavours and off-colours in raw vegetables, plants and seeds (Burnette, 1977).

In contrast to these oxidative enzymes, superoxide dismutase (EC 1.15.1.1) is an antioxidative enzyme that can scavenge singlet oxygen species and transfer them into less active hydrogen peroxide which can be further reduced to less reactive substances such as water and oxygen (Bowler, Montagu, & Inze, 1992). In lentil, the concentration of superoxide dismutase is high in the seed coat and its activity has been reported to be higher than faba bean, pea and horse pea (Troszynska & Kubicka, 2001). Glutathione reductase (glutathione-disulfide reductase, EC 1.8.1.7) is another type of antioxidative enzyme that catalyzes conversion of oxidized glutathione back to reduced form glutathione, which is considered as the most abundant redox couple along with ascorbate in plant cells (May, Vernoux, Leaver, Montagu, & Inzé, 1998; Noctor & Foyer, 1998). Glutathione reductase protects cells from oxidative damage caused by reactive oxygen species under high stress conditions, such as exposure to metal-metalloids, salinity condition and drought stresses (Gill et al., 2013). All of these enzymes work together and play important roles in plant defense mechanisms, especially during harsh conditions (Bowler et al., 1992).

2.1.1.2 Proteins as antioxidants

Proteins in legumes play an important role in antioxidant activity besides contributing to the nutritive value. Fernandez-Orozco, Zieliński and Piskula (2003) investigated four lentil varieties (*var.* Agueda, Almar, Paula and Alcor) and showed that the soluble proteins (79.8 to 132.1 mg/g) contribute to the total antioxidant activity of water extracts. The reason that proteins contribute to

antioxidant capacity is several types of amino acids can donate protons in aromatic residues to electron deficient radicals, scavenge free radicals or chelate metal ions (Arcan & Yemenicioğlu, 2007). Although all 20 biologically derived amino acids retain the capacity to be oxidized, the most reactive amino acids tend to be those containing either nucleophilic sulfur-containing side chains (cysteine and methionine) or aromatic side chains (tryptophan, tyrosine and phenylalanine) from which hydrogen is easily abstracted (Elias, Kellerby, & Decker, 2008). Another reason for the antioxidant activity of protein is that phenolic compounds and proteins can form phenol-protein interactions and such interactions exhibit high antioxidant capacity (Tsai & She, 2006).

2.1.2 Carbohydrates

Total carbohydrate content makes up over 60% of dry weight of raw lentil (Berrios, Morales, Cámara, & Sánchez-Mata, 2010). Starch and insoluble dietary fibre account for about 40% and 10%, respectively of raw lentil's dry weight (Tovar, Bjoerck, & Asp, 1990). The total oligosaccharide content in lentil ranges from 95.5 mg/g to 122.9 mg/g and raffinose, ciceritol and stachyose are the major oligosaccharides of lentils while verbascose is a minor one in lentils (Han & Baik, 2006). Other soluble sugars include sucrose, fructose, glucose, maltose, etc. (Berrios et al., 2010; Martín-Cabrejas et al., 2006). Table 2.2 shows the sugar composition of insoluble dietary fibre and soluble carbohydrates.

Table 2.2 Sugar composition of insoluble dietary fibre and soluble carbohydrates of lentil seed

Type	Sugars and components	Amount (g/100 g dry sample) ^a	Amount (g/100 g dry sample) ^b
Insoluble dietary fibre	Glucose	7.77	
	Xylose	1.27	
	Galactose	0.10	
	Arabinose	2.60	
	Mannose	0.13	
	Uronic acid	0.97	
	Klason lignin	4.12	
Soluble carbohydrates	Ribose		0.31
	Fructose	0.11	0.09
	Sucrose	0.92	0.70
	Maltose	0.05	0.04
	Raffinose	0.22	1.21
	Ciceritol	1.05	2.25
	Stachyose	1.64	

^a Martín-Cabrejas et al. (2006)

^b Berrios et al. (2010)

2.1.3 Lipid and fatty acids

The lipid content in lentil is relative low (1.30 - 1.59%) when compared with chickpea (5.64 - 6.14%) or soybean (20.10 - 22.30%) (Boschin & Arnoldi, 2011). However, the lipid content in lentil seed varies by cultivar. Zhang et al. (2014) investigated 20 Canadian lentil cultivars (10 red and 10 green) and found that the lipid content range from 1.52 - 2.95% for the 10 red lentil cultivars and from 1.69 - 2.63% in the 10 green cultivars. The variation could come from the cultivar, the geographical location (Zhang et al., 2014) and even the storage condition (Sravanthi, Jayas, Alagusundaram, Chelladurai, & White, 2013). The composition of major fatty acids of lentil lipids is shown in Table 2.3. The dominant fatty acid in lentil seed oil fraction is C18:2, followed by C18:1, C16:0 and C18:3.

Table 2.3 Composition of major fatty acids of lentil seed

Fatty acids	% of fatty acid ^a	% of fatty acid ^b	
		Red	Green
14:0	0.4	0.14 - 0.33	0.21 - 0.35
16:0	17.9	13.19 - 14.82	12.67 - 14.76
18:0	2.0	1.13 - 1.73	1.02 - 1.44
18:1	20.1	20.11 - 27.25	21.17 - 28.00
18:2	37.6	40.73 - 47.06	40.97 - 45.46
18:3	6.9	9.32 - 13.28	9.00 - 11.86
Others	15.1		

^a Grela & Günter (1995)^b Zhang et al. (2014)

2.1.4 Phenolic compounds

Besides the important macronutrients, various bioactive phytochemicals in lentil seeds have been studied and evaluated by researchers. These include phenolic compounds, phytosterols, phytic acid, saponins, lectins, defensin and protease inhibitor (Faris et al., 2013). Some of them such as phenolics, may affect protein digestibility and mineral bioavailability (Amarowicz & Pegg, 2008). However, these compounds are also thought to reduce the incidence of various types of cancer, low density lipoprotein (LDL) cholesterol, type-2 diabetes and heart disease (Roy et al., 2010). Phenolic compounds such as flavonoid, lignin and tannin have antioxidant properties which contribute to the nutritional and technological importance of lentil (Amarowicz & Pegg, 2008).

Phenolic compounds provide high antioxidant activity, indicating they are the major antioxidants in lentil (Oomah, Caspar, Malcolmson, & Bellido, 2011). Red lentil contains the highest total phenolic content (TPC) (4.86 - 9.60 mg gallic acid eq. mg GAE/100 g) among eight legumes that include green pea (0.65 - 0.95 mg GAE/100 g), yellow pea (0.85 - 1.14 mg GAE/100 g), chickpea (0.98 mg GAE/100 g), yellow soybean (1.57 - 1.74 mg GAE/100 g), black soybean (5.57 mg GAE/ 100 g), red kidney bean (4.05 mg GAE/100 g) and black common bean

(0.57 - 6.99 mg GAE/100 g) (Xu & Chang, 2007). Some of the major phenolic compounds in lentil (*var.* Morton) include kaempferol, catechin glucoside, digallate procyanidin, procyanidin tetramer, and flavonoid derivatives (Zou, Chang, Gu, & Qian, 2011). Because of the high content and wide variety of phenolic compounds, the antioxidant activity of lentil is relatively high. Red lentil has higher antioxidant activity in comparison with green pea, yellow pea, chickpea, yellow soybean, black soybean and red kidney bean when measured for 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity and ferric reducing antioxidant power (FRAP) (Xu & Chang, 2007). The antioxidant activity measured as Trolox equivalent antioxidant capacity (TEAC) for lentil (*var.* Pardina and Crimson) was higher than other legume seeds including chickpea, yellow pea, green pea and soybean (Han & Baik, 2008). Also, other measurements such as FRAP and total radical-trapping antioxidant parameter (TRAP) were conducted and the results indicated a higher antioxidant activity for lentil compared with bean, broad bean, chickpea and pea (Pellegrini et al., 2006).

The distribution of phenolic compounds in lentil seed differs between the cotyledon and seed coat. According to Tiwari & Singh (2012), generally, the non-flavonoid phenolic compounds are mainly located in the cotyledon, while flavonoids are found in the seed coat. Although the weight proportion of seed coat is lower (8.2 - 11.4%) than that of cotyledon (88.6% - 91.8%), catechins, procyanidins, flavonols and flavones in seed coat are the main contributors to the total phenolic content of lentil (*var.* Pardina and Castellana), whereas cotyledons only provide a small proportion of phenolic compounds, mainly cinnamic and benzoic derivatives (Dueñas, Hernández, & Estrella, 2002).

2.1.5 Vitamins

Vitamin E (tocopherol) is a fat soluble antioxidant and exists mainly in eight different forms, which are α -, β -, γ - and δ -tocopherol, and four types of tocotrienols (Boschin & Arnoldi, 2011). These compounds are able to scavenge free radicals so as to interrupt the chain reactions that are

responsible for the peroxidation of unsaturated lipids (Bramley et al., 2000). Table 2.4 summarizes the content of tocopherols present in lentil seeds and no tocotrienols have been detected.

Table 2.4 Contents of tocopherols in lentil seed

Types of tocopherols	Amount (mg/100 g dry sample) ^a	Amount (mg/100 g wet sample) ^b
α -	0.38 - 0.87	0.25 - 0.40
β -	0.19 - 0.38	--
γ -	9.11 - 10.47	3.72 - 5.00
δ -	0.20 - 0.27	0.05 - 0.06

^a Fernandez-Orozco et al. (2003)

^b Boschini & Arnoldi (2011)

Lentil is not a rich source of Vitamin C (ascorbic acid), which is a water soluble antioxidant commonly found in fruits and vegetables (Faris et al., 2013). In lentil seeds, vitamin C is determined as ascorbic and dehydroascorbic acid. According to Hsu, Leung, Finney and Morad (1980), the content of vitamin C in raw lentil seeds is 0.9 mg/100 g dry sample.

2.2 Heat treatment on legume seeds

Although lentil is a good source of macronutrients, micronutrients and antioxidants, raw lentil need a relatively long cooking time to make the seeds palatable to human consumption. Therefore, application of raw lentil as an ingredient in the food industry is limited (Arntfield et al., 1997; Bellido, Arntfield, Scanlon, & Cenkowski, 2003). It is reported that at least 30 min is needed to cook lentil seed (*var.* Laird) to reach a fully cooked stage and the cooking time can vary from 30 min to 70 min depending on the method used (Cenkowski & Sosulski, 1997). Hence, a precooking process to reduce cooking time is needed in the development of this food ingredient. To solve this, various types of heat treatment can be employed to the whole seed, such as boiling, pressure cooking and microwave cooking.

The benefits of heat treatment involve removal or significant reduction of antinutrients, such as trypsin inhibitor, phytic acid and tannins. Heating treatment can also improve mineral bioavailability, protein digestibility, sugar availability and crude fibre amount which are beneficial for the human body (Satya, Kaushik, & Naik, 2010). Heat treatment with or without the presence of water can cause a breakdown of cell structure, causing an improvement in digestibility. A decrease in cellulose, hemicellulose and lignin content of lentil seed occurred under several types of heat treatments including ordinary cooking for 60 - 120 min, pressure cooking for 5 - 15 min at 103.421×10^3 Pa, or microwave cooking for 4 - 10 min after 4 h soaking in water (Rehinan, Rashid, & Shah, 2004). Heat treatment also improves the sugar availability. Soluble fibre content of lentil seed increased from 3.75% to 7.65% while insoluble fibre decreased from 31.36% to 16.63% after boiling for 3 h (Candela et al., 1997). Soaking at 20 °C for 16 h followed by boiling for 30 min induced an increase in available starch and a decrease in resistant starch of lentil seed (*var.* Pardina) (Aguilera, Esteban, Benítez, Mollá, & Martín-Cabrejas, 2009). Cooking 20 min and autoclaving at 121 °C for 7 min caused an increase in rapidly digestible starch and a decrease in slowly digestible starch. Heat treatment also induces the hydrolysis of carbohydrate. A soaking process at 28 - 42 °C for 60 min produced a general increase in glucose and fructose content (22 - 967%) in lentil (*var.* Vulgaris) (Vidal-Valverde et al., 2002).

On the other hand, common heat treatments including water boiling and pressure cooking may cause physical losses in water soluble protein, starch, vitamins, minerals and oligosaccharides (Satya et al., 2010). Some of the enzymes are highly heat resistant, such as peroxidase and lipoxygenase and can be used as an indicator of adequacy of heating (Yemenicioğlu, Özkan, Velioğlu, & Cemeroğlu, 1998). Antioxidants including phenolic compounds may also be affected upon heat treatment, involving the destruction of old compounds and formation of new compounds (Nayak, Liu, & Tang, 2015). Heat treatment can also induce Maillard reaction in lentil seeds, producing various products and causing browning (Erbersdobler

& Somoza, 2007). Overall, heat treatment can bring benefits to lentil mainly in deactivation of oxidative enzymes but has a complicated effect on antioxidants and antioxidant activity.

2.2.1 Infrared heat treatment (Micronization)

Infrared heat treatment (micronization) is a process employing infrared radiation to precook grains (cereals and legumes) continuously before final use for animal feed, human food and other intentions. This process utilizes electromagnetic radiation from wavelengths of 1.8 - 3.4 μm in order to dry heat the materials that are exposed to it (Zheng, Fasina, Sosulski, & Tyler, 1998). In one study, lentil seed (*var.* Laird) after infrared heating to internal temperatures of 138 and 170 °C showed lower force to compress compared with the control group, indicating a shortened cooking time can be applied (Arntfield et al., 2001). Actually, by utilizing infrared heat treatment, cooking time was shortened from 30 min to 15 min for the lentil seed (*var.* Laird), and 10 min for the ones with higher initial water content (Cenkowski & Sosulski, 1997).

Infrared heat treatment not only decreases cooking time of legume seed, but changes texture properties as well. After infrared heating, increases in percentage of starch gelatinized and decreases in protein solubility, phytic acid and neutral detergent fibre in lentil seeds (*var.* Laird) (Arntfield et al., 2001) were found. Also, infrared heat treatment affected cell wall strength and fracture occurred along the cell walls, not through cell walls (Arntfield et al., 2001). These changes in textural properties imply a softer texture of lentil seed. Also, infrared heating elevated the water holding capacity and oil holding capacity of lentil flour (*var.* Eston) (Pathiratne, 2014). Effects of infrared heat treatment can also be observed from other legumes. Following infrared heat treatment, cowpeas showed softer texture than raw ones due to fissures in the seed coat and cotyledon which was caused by infrared radiation (Mwangwela, Waniska, & Minnaar, 2006).

2.2.2 Effect of heat treatment on phenolic compounds

Under heat treatment such as infrared heat treatment, not only the content, but also the activity of antioxidants in lentil and other legumes may change, compared with the raw seed (Xu

& Chang, 2009). Several types of heat treatments including boiling, steaming, pressure boiling and pressure steaming may cause losses in the contents of total phenolics (49.3 - 62.1%), procyanidins (45.3 - 61.7%), total saponins (7.3 - 34.6%), and phytic acid (15.0 - 21.9%) while the chemical antioxidant capacities in terms of ferric reducing antioxidant power (FRAP) (58.6 - 68.5%) and peroxy radical scavenging capacity (34.8 - 68.7%), and the cellular antioxidant activity of the lentil seed (*var.* CDC Richlea) were also lowered (Xu & Chang, 2009). The main reason for such high material loss could be due to the use of water as the heating media during the thermal treatments and subsequent removal of liquid material. However, pre-treatments such as soaking, boiling and steaming have different effects on antioxidant content and antioxidant activity of different types of legumes (green pea, yellow pea, chickpea and lentil). For example, under 121 °C and 103 kPa pressure cooking, extracts from common beans contained a lower amount of phenolic compounds than raw seeds, but the total antioxidant activity (including cooking water, seed coat and cotyledon) evaluated by DPPH method was higher than the raw ones, because the cooking water exhibited much more antioxidant activity (Rocha-Guzmán, González-Laredo, Ibarra-Pérez, Nava-Berúmen, & Gallegos-Infante, 2007). In another study, after 125 °C dry heating for 25 min, the antioxidant activity of moth bean measured by DPPH radical scavenging activity, ABTS cation radical scavenging activity and ferric reducing antioxidant capacity decreased while the hydroxyl radical scavenging activity did not change (Siddhuraju, 2006). Researchers also found that total phenolic content, DPPH free radical scavenging activity of legumes (green pea, yellow pea, chickpea and lentil (*var.* CDC Richlea)) decreased after soaking, boiling or steaming, but pressure boiling and pressure steaming increased ORAC values and steaming treatment increased TPC, DPPH and ORAC values (Xu & Chang, 2008). It is also found that soaking and draining of liquids adversely affected total phenolic compounds and antioxidant capacity measured by DPPH assay, but cooking procedures without soaking and discarding water help to increase the total phenolic compound levels and antioxidant activity in Brazilian beans (Ranilla, Genovese, & Lajolo, 2009). For several common

phenolic compounds including catechins, procyanidins, flavonols, flavones and flavanones, a decreasing trend was found after processing, but the level of hydroxybenzoic compounds increased in lentil (*var*: *Pardina*) after the same thermal processing (Aguilera et al., 2010).

All of these results suggest that the contents of antioxidants extracted from legumes decrease after cooking, steam cooking, or other industrial processing, but antioxidant activity does not follow a simple trend, some decrease, some increase, while others stay the same (Akillioglu & Karakaya, 2010; Wolosiak et al., 2010; Wolosiak et al., 2011). One proposed explanation for this phenomenon is that different solvents were used by researchers to extract compounds with antioxidant activity, such as water, acetone, and methanol, etc., and the effects of solvent on extractability of components vary largely (Xu & Chang, 2007). After heat processing, the solubility of non-phenolic antioxidants is probably improved and this may contribute to the unchanged total antioxidant activity determined by free radical scavenging ability (Aguilera et al., 2010). According to Nayak et al. (2015) most likely, new compounds with antioxidant activity can be produced during heat treatment while old ones are destroyed.

2.2.3 Effect of heat treatment on antioxidant activity of proteins

The effects of thermal treatments on antioxidant activity of legume proteins have been studied by several researchers (Elias et al., 2008; Arcan & Yemenicioğlu, 2007), and the proteins in lentil are another source of antioxidants (Fernandez-Orozco et al., 2003). In one study, antioxidant activity measured by TEAC value of pea protein isolate showed an increase after heating at 90 °C in an oven for 3 min (Žilić, Akilloğlu, Serpen, Barać, & Gökmen, 2012). The superoxide anion capturing rate and reducing power (OD value) of chickpea protein hydrolysates increased after high pressure treatment (100 - 300 MPa) (Zhang, Jiang, Miao, Mu, & Li, 2012). After thermal processing (121 °C for 20 min) or heat treatment (90 °C for 20 min), water soluble protein extracts from chickpea and white beans exhibited higher free radical scavenging capacity than those of raw ones (Arcan & Yemenicioğlu, 2007). Elias et al. (2008) concluded that the

disruption of tertiary structure of proteins can increase the solvent accessibility of oxidatively labile amino acid residues and consequently increases the protein's overall antioxidant activity. Therefore, both heat treatment and enzymatic hydrolysis may promote the antioxidant activity of proteins.

2.2.4 Effect of heat treatment on enzymes

Heat treatment induces a decrease in the activities of peroxidase and lipoxygenase which are responsible for catalyzing lipid oxidation (Akyol, Alpas & Bayındırlı, 2006). These two enzymes can be used as indicators of whether heat treatment is applied sufficiently since they are heat resistant (Yemenicioğlu et al., 1998). It is found that the deactivation of peroxidase and lipoxygenase follows first-order kinetics, which means that with time, the activity curve has a sharp decrease at the beginning followed by a much slower decrease at later stage (Yemenicioğlu et al., 1998). Similar deactivation patterns were found for other legume species. Ninety percent of lipoxygenase activity of green beans was deactivated within first 10 min at 60 °C of blanching, but it took another 20 min to deactivate the remaining activity (Bahçeci, Serpen, Gökmen, & Acar, 2005).

There are varying effects of heat treatment on antioxidative enzymes. A mild elevated temperature can increase activities of catalase, superoxide dismutase and ascorbate peroxidase which are associated with the self defence against lipid oxidation (Chakraborty & Pradhan, 2011). It is also found that superoxide dismutase from pea is heat resistant and phenolic compounds play roles to enhance the heat resistant stability and antioxidant capacity of pea superoxide dismutase after 70 °C heating for 8 h (Tsai & She, 2006). Consequently, heat treatment seems an ideal pre-treatment to deactivate oxidative enzymes while keeping the activities of antioxidative enzymes.

2.2.5 Maillard reaction products

The Maillard reaction is a series of chemical reactions between reducing sugars and amino compounds (Yilmaz & Toledo, 2005). It can be considered as one of the most important reactions

taking place during food processing because cooking processes such as roasting, baking or frying rely on favorable effects of the Maillard reaction such as colour and flavour formation of food (Jaeger, Janositz, & Knorr, 2010). Maillard reaction products, such as melanoidins have been reported to have antioxidant activity through scavenging oxygen radicals or chelating metals (Yilmaz & Toledo, 2005).

Both the contents of carbohydrate and protein are high in lentil flour (Faris et al., 2013). Thus, heat treatment may hydrolyze protein and carbohydrate and in the presence of reducing sugars, amino acids can react with each other in heated condition and produce Maillard reaction products that are antioxidative. In one study of pea flour, furosine, an indicator of Maillard reaction products, was found in pea protein isolates and a positive correlation between TEAC value and furosine content was shown for the thermally treated samples (Žilić et al., 2012).

2.3 Lentil flour as a meat binder

Lentil flour can be used in meat products to decrease the cost and to increase protein content in order to perform some textural benefits to meat products. This is because lentil seed contains high amount of proteins which exert functions in water holding and oil absorption. The water holding capacity (WHC) of lentil protein concentrate is approx. 4 mL/g and the oil absorption capacity of lentil protein concentrate is 1 - 2 g/g (Boye et al., 2010). The starch in lentil also contributes to the total water holding capacity of lentil flour with 0.98 mL/g of WHC (Hoover, & Sosulski, 1986). Therefore, lentil flour has been investigated as a binder in raw meat products. Baugreet, Kerry, Botineştean, Allen and Hamill (2016) added 7% (w/w) lentil flour into beef patties and found that the hardness of the cooked product was decreased from 79 N to 69 N while the cooking loss was decreased from 26% to 10%. In another study, adding lentil flour and chickpea flour at 6% (w/w) to beef patties caused a decrease of cooking loss from 42% to approx. 22%, a decrease in drip loss from 8% to approx. 2% and a decrease in shear force from 10 N to approx. 7 N (Shariati-Ievari et al., 2016). Also, beef meat balls which were extended with lentil

flour at 10% (w/w) level showed higher cooking yield, higher moisture retention and lower diameter reduction than those with added black bean flour (Serdaroğlu, Yıldız-Turp, & Abrodímov, 2005). In a previous study conducted in our lab (Der, 2010), it was also found that incorporating lentil flour improved textural properties of beef burgers.

Maintaining sensory quality is a critical factor when applying lentil flour as a binder into meat products. The results of sensory evaluation showed that higher sensory scores (juiciness and texture), and higher overall acceptability scores were given to burgers with 6% (w/w) added lentil flour compared with the all meat control burgers (Der, 2010). However, for Der's work (2010), no significant differences in the flavour intensity or aroma were found between the burgers with added lentil flour and the control. Also, adding infrared heat-processed lentil flour into beef burgers caused a positive effect on flavour acceptability compared with the control burger, possibly due to the less off-flavour produced. Therefore, lentil flour could be a potential alternative to the other meat binders, such as toasted wheat crumb and soy protein products. All the nutritional properties and functional benefits make lentil flour a potential binder for meat products.

2.4 Meat colour

The main topics to consider for the properties of meat products other than texture include colour characteristics, lipid oxidation and shelf life. Colour is one of the most critical properties when selecting fresh raw meat and raw meat products, because consumers apply colour as an indicator of freshness and wholesomeness of these products (Faustman & Cassens, 1990). It is true though that consumer's eating satisfaction at home might depend only on the quality attributes of cooked meat, such as tenderness, juiciness and flavour once the decision to purchase is made at the point of sale (Troy & Kerry, 2010). Carpenter et al. (2001) found that consumer's preference for meat colour affected their purchase decisions. More importantly, other properties cannot be fully examined at the point of purchase since consumers cannot smell the odours or feel

the texture before opening the package. Any meat sold in the market that cannot reach consumer's expectations will negatively affect its market value (Troy & Kerry, 2010). That is to say that beef and beef products should have a bright cherry red colour, and pork and chicken should have an even pink colour. The meat, however, tends to discolour easily because of the auto-oxidation of its major pigment - myoglobin. Such discoloration of meat may be accelerated in the retailer's display case and after purchase, thus causing losses. Smith, Belk, Sofos, Tatum and Williams (2000) made an estimation that 15% of retail sales in the United States were discounted due to the discoloration of meat and accounts for an annual loss of one billion dollars.

2.4.1 Meat pigment

Myoglobin is the major meat pigment. It consists of a heme prosthetic group and a globin moiety that forms a cavity to enclose and protect the heme molecule and confer water solubility as well (Faustman & Cassens, 1990). In the heme complex, the ferrous iron can form six coordinate bonds, four with the porphyrin ring and one with histidine which connects heme to the globin. The sixth bond is free for binding oxygen or other small ligands such as oxygen (Cornforth & Jayasingh, 2004). The heme group is also an important component of hemoglobin and other hemo-proteins, such as cytochrome, endothelial nitric oxide synthase, and catalase, etc. (Cornforth & Jayasingh, 2004).

Deoxymyoglobin (deoxyMb) is present when no ligand is bound at the sixth coordination site and the heme iron is in the ferrous (Fe^{2+}) form. This results in a purplish-red or purplish-pink colour of meat. Such colour is mainly present in newly slaughtered meat or in deeper part of meat cuts. When myoglobin is exposed to oxygen, characterized by the development of a cherry-red colour, the sixth coordination site is occupied by diatomic oxygen and oxymyoglobin (oxyMb) is formed. This form of myoglobin is the major pigment when aerobically-packaged or high oxygen modified atmosphere packaged meat is sold as it exhibits the cherry red colour that consumers are expecting (Mancini & Hunt, 2005). Oxidation occurs when ferrous iron transforms to ferric iron

under low oxygen partial pressure or during extended storage. At this time, metmyoglobin (metMb) forms and the characteristic colour of meat is brown or brownish red. The myoglobin chemistry in meat has been well documented in the review articles by Mancini and Hunt (2005) and Suman and Joseph (2013).

2.4.1.1 Endogenous metmyoglobin reducing systems of meat

It is believed that metmyoglobin does not accumulate in healthy living muscle, so it is being continuously reduced within the muscle tissue (Arihara, Cassens, Greaser, Luchansky, & Mozdziak, 1995). The metmyoglobin could be reduced to deoxymyoglobin depending on muscle's mitochondrial functions, including oxygen scavenging enzymes, reducing enzyme systems, and the NADH pool (Ramanathan, Mancini, & Maheswarappa, 2010). NADH-Cytochrome *b5* reductase was found to work with outer mitochondria cytochrome *b* in mitochondria and cytochrome *b5* in the sarcoplasmic reticulum as electron transfer mediators to reduce metmyoglobin with the presence of NADH (Arihara et al., 1995). However, after slaughter and with increased time of post-mortem, both the enzyme activity and the NADH pool are continually depleted which results in the oxidation of the pigment and permanent discoloration (Suman & Joseph, 2013). In a model system with mitochondria and metmyoglobin, the addition of 2 mol/L NADH into the system decreased the level of metmyoglobin by 56% compared with the control (Gao, Wang, Tang, Ma, & Dai, 2014). This suggests the importance of NADH to the metmyoglobin reducing system.

2.4.2 Measurement techniques of meat colour

According to the American Meat Science Association (AMSA) meat colour measurement guidelines (AMSA, 2012), visual appraisal and instrumental measurements can be used to measure meat colour. The visual appraisal of meat colour uses human panelists to evaluate meat colour. Instrumental meat colour measurement, alternatively provides an objective way to measure meat colour. With the application of colorimeters and spectrophotometers, meat colour

can be quantified as tristimulus values (CIE L* a* b*) or spectrum in intervals of 1 to 10 nm. When 100% standard meat for deoxy-, oxy-, and met- forms of myoglobin are prepared, the redox forms of myoglobin can be calculated using spectrophotometric values for each myoglobin form at the isobestic wavelengths (474, 525, 572 and 610 nm) (AMSA, 2012; Mancini, Hunt, & Kropf, 2003).

2.4.3 Application of phenolic compounds in raw meat products to protect meat colour

Ground meat or ground meat based raw meat products, such as burgers and patties are widely consumed in North America. A study showed that ground beef accounts for about 15% of supermarket meat department beef sales in the US with a total of 10 billion pounds (American Meat Institute, 2009). However, ground beef discolours much easier than meat cuts since cutting, mixing and grinding are applied and all these processes increase susceptibility to oxidation (Mitsumoto, O'Grady, Kerry, & Joe Buckley, 2005). Researchers tried different ways to protect and stabilize fresh meat colour during packaging, storage and retailing. Suman, Hunt, Nair and Rentfrow (2014) reviewed the strategies investigated by researchers, including animal feeding, packaging technologies and exogenous antioxidants. Among exogenous antioxidants, phenolic compounds or herbal extracts rich in phenolic compounds have been studied to apply in raw meat or raw meat products, such as ground beef or beef patties (Allen & Cornforth, 2010; Vargas-Sánchez et al., 2014; Hayes, Stepanyan, Allen, O'Grady, & Kerry, 2010).

Phenolic compounds are the main class of plant secondary metabolites. In plants, phenolic compounds are part of the chemical defense system to protect against pathogens, parasites and predators besides helping in reproduction and growth of plants (Nayak et al., 2015). Fruits, vegetables, cereals, oilseeds and legumes are the most common food sources of phenolic compounds (Nayak et al., 2015). The dietary phenolic compounds can be absorbed into the human body and act as free radical scavengers and metal ion chelators (Dangles, 2012). In the

human body, they have many health benefits, such as antioxidant, antimicrobial and anti-inflammatory activities (Landete, 2012).

A variety of phenolic compounds or herbal extracts that contain phenolic compounds have been employed in the raw meat products to test their ability to improve meat colour stability and to inhibit lipid oxidation. While some of them were targeted at beef products (Yu, Ahmedna, & Goktepe, 2010), others were applied to pork or chicken (Sáyago-Ayerdi, Brenes, & Goñi, 2009). In our group's previous work, we also found that lentil flour can improve colour stability in postharvest applications of meat (Der, 2010; Pathiratne, 2014). When lentil flour (*var.* Laird and CDC Redberry) was incorporated into beef burgers, the discoloration of burgers slowed down during 4 °C storage and at the end of storage the burgers incorporated with lentil flour had higher a^* values (redness) than those of the control. Interestingly, heat-treated (infrared heating) lentil flour showed more potency in colour improvement of raw burgers in comparison to addition of raw lentil flour (Der, 2010).

2.4.3.1 Forms of phenolic compounds

Single phenolic compounds and plant-based extracts have been studied in meat systems. Single phenolic compounds without other substances or impurities can be easily quantified. The effectiveness of phenolic compounds as a meat colour protector and their activity as antioxidants can be compared to others using indicators and model assays employed by the industry and research. For instance, rosmarinic acid is a type of phenolic compound that is found in a variety of plants (Frankel, Huang, Aeschbach, & Prior, 1996). Allen and Cornforth (2010) tested the effect of adding 0.5% (w/w) of rosmarinic acid on the colour changes of beef patties. The results indicated that rosmarinic acid preserved redness of beef patties (higher a^* value) compared with the control sample during a 14-day storage at 4 °C. Overall, single phenolic compounds either from natural sources or from chemical synthesis give a clear and quantitative effect on colour protection of raw meat products.

Plant-based extracts containing phenolic compounds can also be applied to meat products. Some of the natural extracts are available from commercial sources, such as extracted phenolic compounds from tea and grape seeds. Tea extracts contain a high amount of catechins and grape seed is also a good source of phenolic compounds (Rababah, Hettiarachchy, & Horax, 2004). Mitsumoto et al. (2005) investigated the effects of adding tea catechins (commercial source) on colour of raw beef patties during a 6-day refrigerated storage. In their study, tea catechins (200 and 400 mg/kg meat) were added into beef patties. But for meat colour (a^* value), there was no difference found for tea catechin addition to raw beef burgers versus the control. However, results from other sources are different. Bañón, Díaz, Rodríguez, Garrido and Price (2007) investigated the effect of green tea and grape seed extracts (commercial source) on the colour changes of beef patties during refrigerated storage of 9 days. They added 300 mg/kg (0.03%) water soluble extracts (>30% total catechins and gallic acid) and found that samples with added grape seed and green tea extracts had lower L^* values (lightness), higher C^* values (Chroma) and lower H^* values (Hue angle) during the 9 day storage (4 °C), indicating a colour protecting ability of these extracts. In the work by Liu, Xu, Dai and Ni (2015), grape seed extracts (commercial source) and tea catechins at level of 300 mg/kg (0.03%) were added into raw beef patties and the colour change and lipid oxidation were analyzed during 4 °C storage for 8 days. The results showed that redness (a^* value) of samples with antioxidants were higher than the control on day 8. The metmyoglobin level of treatment samples was also lower than the control. There are big differences in composition, amount, and antioxidant ability of different sources of phenolic compounds (eg. tea catechins, grape seed extracts) obtained by different extracting methods (Nayak et al., 2015), thus causing different levels of effects on meat colour protection and making it difficult to compare across studies.

Herbal extracts could contain a large amount of phenolic compounds and they are important sources of antioxidants (Kähkönen et al., 1999). Phenolic compounds could be extracted from various plant parts, including roots, stems, bark, leaves, fruits and seeds (Shah, Bosco, & Mir,

2014). But they are not always easily accessible, compared with the commercial antioxidant extracts. The extraction process employs different solvents to obtain maximum yield of the antioxidants (Shah et al., 2014). Successful examples include using the extracts of willowherb, a flowering plant that has been traditionally used in folk medicine, on the colour of beef patties (Cando, Morcuende, Utrera, & Estévez, 2014)) and using the extracts of peanut skin in ground beef (Yu et al., 2010). But one thing to consider when using phenolic compounds is that quinones as the oxidation products of phenolic compounds can induce the oxidation of oxymyoglobin and make the meat colour turn brown (Castro, Hathaway, & Havlin, 1977). Another thing to consider is that some other characteristics of the herbal extracts may also influence the colour protection results. For instance, Kim, Cho, and Han (2013) prepared extracts from two leafy green vegetables (*chamnamul* and *fatsia*) by using 70% ethanol (v/v), followed by evaporation and freeze drying. The extracts which contain approximately 23 mg/g total phenolic content was added (0.1% and 0.5 % (w/w), respectively) into beef patties and the patties were stored at 4 °C for 12 days. They found that the vegetable extracts had a particular greenish colour that was transferred to the patties, causing a modification of the original patty colour. This reminds us that when trying to add antioxidants or plant based extracts into meat products, the original colour of the extracts cannot be ignored since it may alter the colour of meat products.

Not all the extracts are transparent, so they may affect meat colour. But understanding the contribution of their colour to the meat colour is complicated. If the extract shows a red colour, it may improve the meat product colour by covering the original meat colour and it is hard to find out whether this color preservation comes from the antioxidant activity of the extract or simply comes from its colour. If the colour of the extract is green, brown or any dark colour, it may make the colour of product worse right after manufacture. This becomes a challenge when considering the concentration of extract added since the colour protection is concentration based. If more extract is added, the antioxidant activity and the colour protection may be raised, but the meat colour may also be affected more; while if less extract is added, the meat colour may not be

affected a lot, but the antioxidant activity may not be high enough to protect meat colour. Some researchers use the colour change (ΔE) or the change of a^* value (redness) to express the change of colour rather than only using the a^* value or Chroma (Cando et al., 2014; Ganhão, Estévez, Kylli, Heinonen, & Morcuende, 2010, 2010; Kim et al., 2013). It is also highly recommended to report the L^* a^* b^* values of the extracts or to describe the colour of extracts, because this would help readers understand the effect of the extracts' colour.

2.4.3.2 Concentration differences

The effect of adding phenolic compounds on meat colour protection is concentration dependent. On one hand, no antioxidant effect or meat colour protection effect would be expressed if too little is added. In one study, Vatter and Shetty (2005) investigated the effects of ellagic acid and sesamol on the colour retention of raw beef patties during a 12-day storage at 4 °C. They found that although samples with added ellagic acid (300 and 600 µg/g muscle) and sesamol (250 and 500 µg/g muscle) lowered lipid oxidation, these antioxidants cannot improve the colour of beef burgers. This is probably because this study used lower dosage (0.05% at most) compared with the previous one using a higher level of rosmarinic acid (0.5%, by Allen and Cornforth (2010)). In another study, extracts from willowherb at levels ranging from 50, 200 to 800 ppm added into beef patties cannot preserve meat colour, as they observed the redness of treatment samples were lower than the control throughout the storage (Cando et al., 2014). Bekhit, Geesink, Ilian, Morton and Bickerstaffe (2003) compared the effects of resveratrol, quercetin and rutin (110 and 550 µmol/kg meat) on the colour of raw beef patties stored at 2 °C for 9 days. The percentage of these antioxidants in patties was 0.003% and 0.013% for resveratrol, 0.003% and 0.013% for quercetin and 0.007% and 0.034% for rutin. They found that all the treatments showed higher Chroma values (C^*) than the control on day 9. Among all samples, beef patties with resveratrol at the level of 550 µmol/kg had the highest Chroma value and the lowest metmyoglobin level on day 9, indicating its better colour retention ability. Peanut

skin extracts at 0.08% and 0.1% (w/w) added into raw ground beef preserved the redness for 12 days, but not at lower levels (0.02 - 0.06%) (Yu et al., 2010).

On the other hand, if extracts with phenolic compounds are added above a certain level, they may not exert more antioxidant capacity or meat colour protection. Jia, Kong, Liu, Diao and Xia (2012) investigated the antioxidant activity and anthocyanin content of black currant extract. The black currant extract was added at levels of 5, 10 and 20 g/kg meat into pork patties and black currant extract at all levels significantly increased the redness of the pork patties, however no difference was found among these levels. As such, there is likely a threshold that exists for antioxidants/phenolic compounds to effectively act as a protector of meat colour in raw meat systems. There is also a maximum level that exists such that if more antioxidant is added, no further benefit can be obtained.

2.4.3.3 Comparison of natural phenolic compounds with other antioxidants

There are other types of antioxidants that have been tested as references on meat colour preservation abilities along with the natural phenolic compounds. These antioxidants included Vitamin C (Mitsumoto et al., 2005; Vargas-Sánchez et al., 2014), BHA (Jia et al., 2012; Liu et al., 2015; Yu et al., 2010), BHT (Kim et al., 2013; Muthukumar et al., 2014; Naveena et al., 2013; Xu et al., 2010; Yu et al., 2010). Comparisons between the natural phenolic compounds and these compounds have been studied. For instance, adding 5 g/kg of black currant extract into pork patties showed higher a^* values than patties added with 0.2 g/kg BHA (Jia et al., 2012). In another study, beef patties added with tea extract and grape seed extract at 0.3 g/kg displayed lower a^* value during storage, compared with beef patties formulated with Vitamin E at 0.3 g/kg or BHA at 0.03 g/kg (Liu et al., 2015). Also, adding 0.02% to 0.10% pea skin extract did not cause higher a^* values compared with 0.02% BHA added into beef patties (Yu et al., 2010). It is possible that these natural phenolic extracts contain some impurities, making them less active than pure standards, such as BHA and BHT.

2.4.3.4 Antioxidant effect of phenolic compounds on raw meat products in frozen storage

Frozen storage is a common way to store meat products since the low temperature can slow down the physical and biochemical reactions that may lead to the deterioration of foods (George, 1993). As a result, the shelf-life of frozen meat products can be prolonged dramatically compared with the refrigerated ones (Leygonie, Britz, & Hoffman, 2012). However, during frozen storage, meat products may also suffer some changes in quality, such as loss of moisture, denaturation and oxidation of proteins and loss of tenderness (Leygonie et al., 2012). In addition, endogenous antioxidative enzymes of meat, such as superoxide dismutase, catalase and glutathione peroxidase had lower activities as a result of frozen storage for ten weeks (Lee, Mei, & Decker, 1997). The myoglobin and lipid oxidation of meat products occur during the frozen storage as well, leading to the discoloration and off-flavour of meat products (Brewer & Wu, 1993).

Researchers have utilized phenolic compounds as antioxidants to prevent the quality loss of meat products induced by myoglobin and lipid oxidation under frozen conditions. In one study, rosemary extracts and alpha tocopherol with chitosan added into beef burgers resulted in a greater a^* values than the control during 180-day frozen storage at $-18\text{ }^{\circ}\text{C}$ (Georgantelis, Blekas, Katikou, Ambrosiadis, & Fletouris, 2007). In another study, Akarpat, Turhan and Ustun (2008) added myrtle extracts, containing phenolic compounds into beef patties and stored them at $-20\text{ }^{\circ}\text{C}$ for 120 days. The treatment with the addition of myrtle extracts showed higher a^* values than the control, suggesting a colour protection effect for beef patties stored under frozen condition. But the long period of frozen storage makes it less convenient to evaluate the antioxidant effect of phenolic compounds on meat colour protection, compared with refrigerated storage.

2.4.4 Myoglobin model system

As the major pigment of meat, myoglobin has been studied in model systems aiming to simplify effects of factors that cause undesirable impacts. A typical myoglobin model system may consist of myoglobin which is dissolved in a buffer solution, target substance, and/or iron as an

accelerator of oxidation (Allen & Cornforth, 2009; Allen & Cornforth, 2006). The objectives of studies relying on myoglobin model systems include the antioxidant or pro-oxidant activity of the target substance and their effect on myoglobin forms (Allen & Cornforth, 2006). The time period of such a system can vary from 2 to 96 h, depending on the different objectives (Gorelik & Kanner, 2001a; Yin et al., 2011). An example is that Allen and Cornforth (2006) studied the effect of iron-chelating agents on oxidation of myoglobin in the presence of non-heme iron and found that chelating agents can help to inhibit myoglobin oxidation. In another study, the same group of researchers evaluated the effects of two types of antioxidants on the oxidation of myoglobin and found that the type II antioxidants (metal chelators) exhibited more potent antioxidant ability than type I antioxidants (radical quenchers) (Allen & Cornforth, 2009). For this type of study, temperature is an important factor that affects the pace of myoglobin oxidation. In the study to investigate the effect of 4-hydroxy-2-nonenal (HNE) on oxidation of myoglobin, researchers used 25 °C and 4 °C as reaction temperatures and the result proved that the reaction is slower under lower temperature (Yin et al., 2011). Myoglobin model systems are built on the fact that different myoglobin forms exhibit characteristic absorbance within the visible region, which lets researchers monitor the content change of different forms of myoglobin (Bowen, 1949). Absorptions scanned from 450 nm to 650 nm can be used to determine the change of myoglobin oxidation (Gorelik & Kanner, 2001b) and this is the most common way to calculate the content of different myoglobin species. Consequently, three major factors need to be considered when applying a myoglobin model system, that is the composition, temperature and storage time. The simplicity of a myoglobin model system makes it a good application to verify antioxidant activity related to myoglobin oxidation.

2.4.4.1 The prevention of myoglobin oxidation by phenolic compounds

The effect of phenolic compounds on the reduction of oxidized forms of myoglobin has been investigated by researchers *in vitro*, mostly, in myoglobin model systems. Masuda, Inai, Miura, Masuda and Yamauchi (2013) investigated the effects of various plant phenolics on the oxidation

of oxymyoglobin using a model system. At concentrations of 0.1 and 1 mmol/L of each phenolic and 60 $\mu\text{mol/L}$ of oxymyoglobin, the phenolic compounds performed as pro-oxidation reagents. The reason that phenolics exhibit prooxidative activity is probably because quinones or semiquinone radicals were formed under oxidative conditions. They also found that cysteine from concentrations of 0.1 mmol/L to 4 mmol/L can inhibit such oxidation promoting the activity of the phenolic compounds because the thiol group in cysteine can interact with quinone and semiquinone radicals to inhibit the prooxidative activity. In another study, Stolze and Nohl (1995) also found that with concentrations ranging from 100 $\mu\text{mol/L}$ to 2 mmol/L, a synthetic phenolic compound butylated hydroxyanisole (BHA) showed oxidative activity when reacting with 0.83 mmol/L oxymyoglobin to form metmyoglobin. With higher concentrations of BHA, the rate of the formation of metmyoglobin became faster. It is therefore suggested that phenolic compounds may not prevent the oxidation of oxymyoglobin. But there is some evidence that they reduce metmyoglobin to oxymyoglobin. Researchers tested the effect of various phenolic compounds (at levels of 300 and 600 $\mu\text{mol/L}$) on the reduction of metmyoglobin to oxymyoglobin in the presence of cysteine (at levels of 300 and 600 $\mu\text{mol/L}$) (Inai, Miura, Honda, Masuda, & Masuda, 2014). They found that phenolic compounds indeed can reduce metmyoglobin and as a result, increase oxymyoglobin level in the model system.

Among phenolic compounds, different structures showed different antioxidant activities. For flavonoids, the dihydroxy structure in the B ring, 2,3 double bond in conjugation with a 4-oxo function in the C ring, and 3- and 5-OH groups with 4-oxo function in A and C rings contributed to higher antioxidant activity (Rice-Evans, Miller, & Paganga, 1996). For phenolic acids, the antioxidant activity depends on the number of hydroxy groups (Rice-Evans et al., 1996). For condensed tannins, which are mostly polymers, they perform better than simple phenolic compounds (Hagerman et al., 1998). Such differences in molecular structure caused different antioxidant activities of phenolic compounds. Specifically, for metmyoglobin reducing activity, higher abilities were found for sinapic acid, kaempferol, myricetin, quercetin and catechin which

contain more hydroxyl groups, in contrast to luteolin, syringic acid, resveratrol and gentisic acid which contain less number of hydroxyl groups and lowered abilities (Inai et al., 2014).

Several other studies analyzed the effects of phenolic compounds on hypervalent myoglobins, namely ferrylmyoglobin and perferrylmyoglobin (Laranjinha, Almeida, & Madeira, 1995; Jongberg, Lund, Skibsted, & Davies, 2014). These hypervalent myoglobin species could be present in fresh meat initiating lipid oxidation (Richards, 2013). Ferrylmyoglobin is always prepared by the reaction of metmyoglobin and hydrogen peroxide. Phenolic acids (Laranjinha et al., 1995; Libardi, Borges, Skibsted, & Cardoso, 2011), flavonoids (Jørgensen & Skibsted, 1998), condensed tannins (Hu & Skibsted, 2002; Yin, Andersen, & Skibsted, 2013) and plant extracts (Jongberg et al., 2014) were all tested and shown to be effective at reducing hypervalent myoglobin. Although there are very few studies on the ferryl- or perferryl- forms of myoglobin in meat related systems, the knowledge is still valuable to evaluate the effect of antioxidants, especially phenolic compounds on myoglobin reducing and on meat colour.

Consequently, with high reducing potential, phenolic compounds can reduce perferrylmyoglobin and ferrylmyoglobin to metmyoglobin, and further reduce metmyoglobin to oxymyoglobin. The proposed scheme of the reducing effect of phenolic compounds is outlined in Figure 2.1. Since myoglobin has absorbance within the visible wavelength region, myoglobin could be an indicator for antioxidant activity of potential antioxidants. Researchers developed a method based on this characteristic of myoglobin (409 nm used in the protocol) to test the antioxidant activity of certain extracts and antioxidants (Terashima, Nakatani, Harima, Nakamura, & Shiiba, 2007).

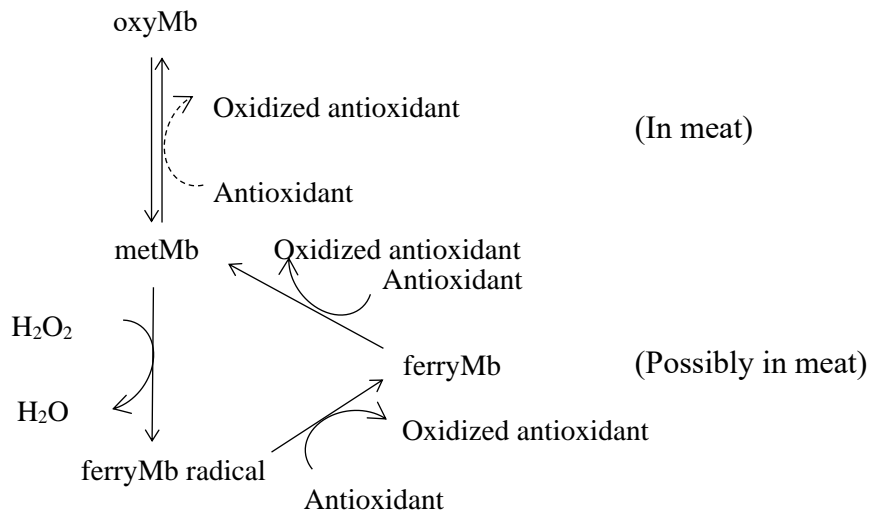


Figure 2.1 Proposed effects of antioxidants on the myoglobin form changes

(Richards, 2013)

2.5 Lipid oxidation of meat

Lipid oxidation is a major factor in meat deterioration and often causes significant loss of quality (Baron & Andersen, 2002). Lipid oxidation in meat is a process in which unsaturated fatty acids, such as oleic acid, linoleic acid, linolenic acid and arachidonic acid react with oxygen to form fatty acyl hydroperoxides via a free radical-chain mechanism (Ladikos & Lougovois, 1990). This procedure (as shown in Figure 2.2a) is initiated when a site on the fatty acyl chain abstracts a labile hydrogen atom to produce a free lipid radical which reacts rapidly with oxygen to form a peroxyradical. The peroxyradical again abstracts a hydrogen atom from another hydrocarbon chain to produce a hydroperoxide and a new free radical which can continue the chain reaction (Ladikos & Lougovois, 1990). The lipid hydroperoxides and their dimers and polymers may decompose to hydroxyl and alkoxy radicals and produces small volatile compounds with unfavorable odours contributing to flavour deterioration (Ladikos & Lougovois, 1990). Flavour deterioration such as warmed over flavour is the result of lipid oxidation in cooked, refrigerated and pre-cooked, frozen meat products (Trout & Dale, 1990). In Der's study (2010), incorporation

of heat-treated (infrared heating) lentil flour (*var.* Laird and CDC Redberry) into beef burgers resulted in lower lipid oxidation during frozen storage at -20 °C over 9 weeks, compared with the non-heated group and the control group. This result combined with the improvement of colour stability implied a higher antioxidant activity of heat-treated lentil flour. Knowledge of the specific antioxidants or mechanisms would enhance optimization of thermally treated lentil flour.

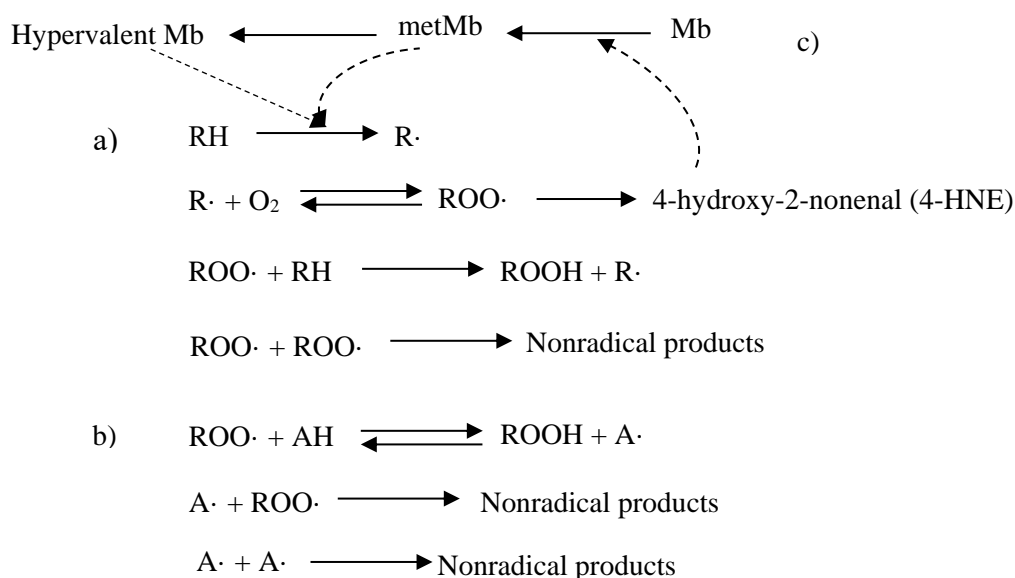


Figure 2.2 a) Lipid oxidation; b) The effects of antioxidants; c) Interaction between myoglobin oxidation and lipid oxidation (4-HNE as the lipid oxidation products is able to accelerate Mb oxidation, while hypervalent Mb as the products of Mb oxidation is able to accelerate lipid oxidation)

2.5.1 Distribution of fatty acids in meat

In meat, fatty acids are distributed in adipose tissue and muscle tissue. In beef, generally, adipose tissue contains more saturated fatty acids, such as stearic acid (C18:0) and palmitic acid (C16:0) and monounsaturated fatty acids, such as oleic acid (C18:1) and palmitoleic acid (C16:1) than polyunsaturated fatty acids (Wood et al., 2008). For pork, adipose tissue (backfat) contains

around 36% saturated fatty acids, 44% monounsaturated fatty acids and 12% polyunsaturated fatty acids (Gandemer, 2002). The triacylglycerols (TAG) represent the major lipid class in adipose tissue (Wood et al., 2008). For muscle tissue, on the other hand, phospholipids (PL) represent 30% of total lipids for cattle at 14 months of age, higher than that in adipose tissue (Wood et al., 2008). But with the increase in animal age, the amount of all types of fatty acids from triacylglycerol lipids keep increasing while the amount of phospholipid fatty acids do not change much (Wood et al., 2008). More importantly, there are more unsaturated fatty acids in phospholipids than in triacylglycerol lipids (Gandemer, 2002). It is believed that phospholipids are the major contributors to the development of rancidity and warmed-over-flavour in cooked meat (Ladikos & Lougovois, 1990). Igene and Pearson (1979) utilized lipid-free muscle fiber as a model system and added triglycerides, total lipids and total phospholipids back into the lipid-free system. They found total phospholipids were the major contributors to the development of warmed over flavour and more TBARS value were found for the systems containing total phospholipids than those containing triglycerides (Igene & Pearson, 1979).

2.5.2 Measurement techniques of lipid oxidation

Various methods are used to determine the degree of lipid oxidation in meat products. The most widely used techniques include peroxide value, hexanal content, thiobarbituric acid reactive substance (TBARS) test and fatty acid volatiles. Several studies used peroxide value to measure the lipid oxidation when applying natural antioxidants (Yu et al., 2010; Sun, Zhang, Zhou, Xu, & Peng, 2010; Badr & Mahmoud, 2011). However, the limitation of this method is that peroxides are intermediate products in the formation of carbonyl compounds, which means they are not stable (Fernández, Pérez-Álvarez, & Fernández-López, 1997). Hexanal content was determined by a few researchers (Jayathilakan, Sharma, Radhakrishna, & Bawa, 2007; Gallego, Gordon, Segovia, & Almajano, 2015; Šulniūtė, Jaime, Rovira, & Venskutonis, 2016). This measurement makes use of steam distillation followed by submitting the volatiles to gas chromatographic analysis. In meat science, the most widely used measurement is the TBARS test (Fernández et al.,

1997). This method is based on the malondialdehyde (MDA) reaction with thiobarbituric acid (TBA) to obtain a red pigment due to the condensation reaction of two molecules of TBA with one molecule of MDA. But this method also has limitations in that MDA and other short chain carbon products of lipid oxidation are unstable for long term storage. Thus, organic alcohols and acids that are produced by these products cannot be determined by the TBARS test (Fernández et al., 1997).

2.5.3 The prevention of lipid oxidation using phenolic compounds

Phenolic compounds are electron-rich aromatic compounds, so they can transfer electrons to reactive oxygen species (ROS) and convert them into aryloxy radical collaterally. The aryloxy radical can be stabilized by delocalization of the unpaired electron over the aromatic nucleus. This function makes phenolic compounds as reducing compounds with high antioxidant activity (Dangles, 2012). The antioxidant functions of phenolic compounds in food rely on several functions: ability to bind metals, ability to inhibit enzymes that produce reactive oxygen species and inhibition effect on lipid autoxidation (Dangles, 2012). Firstly, phenolic compounds generally display more than one site for metal binding. Because transition metal ions, such as Fe^{2+} are typically involved in reactive oxygen species production through oxygen or peroxide hydrogen activation, interactions of metal and phenolic compounds are considered to interfere with the reactive oxygen species forming process. Secondly, polyphenols can also inhibit reactive oxygen species producing enzymes, such as lipoxygenases and NADPH oxidase, because they can develop van der Waals interactions via their aromatic rings and hydrogen bonds through their phenolic groups. Thirdly, phenolic compounds are good inhibitors of lipid autoxidation in phospholipid bilayers due to their favorable interactions with pro-oxidant species. Their facilitated regeneration of α -tocopherol also contributes to its inhibition ability on lipid autoxidation in food system (Dangles, 2012).

Phenolic compounds are typically used in the form of crude extracts from their sources. Such extractions generally follow common operations. Firstly, materials are dried and/or ground and then the ground materials are extracted as much as possible using appropriate solvents, followed by vacuum filtration and concentration using freeze-drying. After that, crude extracts containing high levels of phenolic compounds are added to either ground meat or meat products to evaluate the effects on lipid oxidation and color stability during and after a period of storage (Brettonnet, Hewavitarana, DeJong, & Lanari, 2010; Shirahigue et al., 2010; Yu et al., 2010). In most studies, extracts with high content of phenolic compounds successfully impede lipid oxidation in raw and cooked meat product compared to control groups (Sun et al., 2010; Sáyago-Ayerdi et al., 2009; Vossen, Utrera, De Smet, Morcuende, & Estévez, 2012).

Considering the pro-oxidation effect of phenolic compounds, numerous studies tested different concentrations of natural extractions on lipid oxidation in meat products (Sun et al., 2010; Ali, 2011; Brettonnet et al., 2010; Dejong & Lanari, 2009; López-López et al., 2009; Sáyago-Ayerdi et al., 2009; Shirahigue et al., 2010; Vossen et al., 2012; Yu et al., 2010). For example, 1.00% of *pomposia* extraction incorporated in ground beef had a greater effect than 0.75% and 0.05% on prevention of lipid oxidation (Ali, 2011). Lower concentrations (0.02% and 0.04%) of peanut skin extract also exhibited a smaller antioxidant effect than at higher concentrations (0.06%, 0.08% and 0.10%) in cooked ground beef (Yu et al., 2010).

Researchers utilized different meat systems to test antioxidant abilities of the phenolic extracts of natural sources. These meat systems include ground beef (Ali, 2011), beef and pork sliced ham (Sun et al., 2010), precooked beef, chicken and pork (Brettonnet et al., 2010; Yu et al., 2010), pork emulsion model (López-López et al., 2009), cooked chicken breast burger (Sáyago-Ayerdi et al., 2009), chicken meat ball (Shirahigue et al., 2010), and frankfurters (Vossen et al., 2012). Raw and cooked meat products can exhibit different degrees of lipid oxidation. Typically, lipid oxidation in beef is retarded by antioxidants more successfully than that in pork or chicken (Brettonnet et al., 2010). The degree of lipid oxidation in raw ground beef is lower than that in

cooked ground beef when grape antioxidant dietary fiber is added (Sáyago-Ayerdi et al., 2009). This is probably because the cooking process can damage the muscle cell membrane, so the lipid oxidation could proceed faster in cooked meat. Also, the dissociation of myoglobin and the release of free iron during cooking also can contribute to the higher level of lipid oxidation in cooked meat products.

2.5.4 Myoglobin induced lipid oxidation and the prevention using phenolic compounds

Myoglobin can induce lipid oxidation in raw meat products. This is because free iron can initiate lipid oxidation by producing free radicals capable of abstracting a proton from unsaturated fatty acids (Ramanathan, Konda, Mancini, & Faustman, 2009). All states of iron in myoglobin heme could initiate lipid oxidation in muscle foods. However, the dispute still exists in the mechanisms of myoglobin-induced oxidation/peroxidation of polyunsaturated fatty acids. This is because the mechanism observed under physiological conditions *in vivo* may be different from that occurring in the meat system (Ramanathan et al., 2009).

Myoglobin oxidation in fresh meat and raw meat products have long been connected with lipid oxidation. Faustman et al. (2010) have reviewed the interaction between the oxidation of myoglobin and the oxidation of lipid. Myoglobin works as a facilitator of lipid oxidation (shown in Figure 2.2c) and the products of lipid oxidation, in turn, accelerate myoglobin oxidation (Carlsen, Møller, & Skibsted, 2005). Also the involvement of myoglobin in lipid oxidation in raw meat products has long been linked with hypervalent myoglobin. They are strong pro-oxidants resulting from the reaction of metmyoglobin and peroxides (Yin et al., 2013).

Researchers also investigated the effect of phenolic compounds on myoglobin in the presence of lipid. Vulcain, Goupy, Caris-Veyrat and Dangles (2005) investigated the inhibition of alpha tocopherol and flavonol quercetin on the myoglobin induced peroxidation of linoleic acid in mildly acidic emulsions. The ferrylmyoglobin was prepared by treating metmyoglobin with hydrogen peroxide. Lipid oxidation was monitored by the accumulation of conjugated dienes

(absorbance at 234 nm). They found that alpha tocopherol was an efficient peroxidation inhibitor but was only effective in lipid phase, while quercetin was not only a good lipid oxidation inhibitor, but an efficient ferrylmyoglobin reducer as well. Laranjinha, Vieira, Almeida and Madeira (1996) investigated the metmyoglobin/hydrogen peroxide initiated lipid oxidation occurred in low density lipoprotein (LDL) with the presence of phenolic acids, including *p*-coumaric acid, ferulic acid, caffeic acid, protocatechuic acid, chlorogenic acid and ellagic acid. They found that these phenolic acids can successfully convert ferrylmyoglobin to metmyoglobin and can prevent lipid oxidation by monitoring the perinanic acid oxidation and oxygen consumption. The ferrylmyoglobin formed from myoglobin oxidation accelerated lipid oxidation without the presence of any phenolic compounds. In a similar LDL system with the presence of ferrylmyoglobin, caffeic acid successfully delayed the consumption of alpha-tocopherol, suggesting good antioxidant activity (Laranjinha, Vieira, Madeira, & Almeida, 1995). Others also found that phenolic acids (caffeic acid, ferulic acid and *p*-coumaric acid) can decrease the oxygen consumption of the model system and can reduce ferrylmyoglobin into metmyoglobin (Nakayama, Sato, Kajiya, Kumazawa, & Hashimoto, 2004).

Phenolic compounds can also inhibit the lipxygenase activity of hemoglobin and myoglobin on linoleic acid and linoleoyl alcohol (Goupy, Bautista-Ortin, Fulcrand, & Dangles, 2009). The lipxygenase activity of hemoglobin and myoglobin also has a self-inactivation, in which the addition of substrate (linoleic acid) cannot restore the reaction, which can only be restored by adding fresh hemoglobin (Kühn, Götze, Schewe, & Rapoport, 1981).

2.6 Summary

In summary, lentil flour as a gluten-free ingredient with high phenolic content can be used in meat products and will likely contribute an antioxidant effect to meat products. The protection of fresh red colour of meat products and the inhibition of lipid oxidation can be expected in refrigerated and frozen conditions with the addition of lentil flour. However, the oxidative

enzymes should be deactivated prior to use by applying heat as a pre-treatment to protect unsaturated lipids in the meat from enzyme-induced oxidation. The distribution of antioxidants and enzymes in the lentil physical components are still not well established and the mechanism of how antioxidant components in lentil protect meat colour and inhibit lipid oxidation is still not fully uncovered. Therefore, further study is needed to investigate the antioxidant effect of lentil in meat systems.

3 STUDY 1 ANTIOXIDANT AND ENZYME ACTIVITIES OF WATER SOLUBLE EXTRACTS OF LENTIL SEED COMPONENTS AND THE EFFECT OF HEAT TREATMENT

3.1 Abstract

Lentil is a healthy food high in protein, fibre and phenolic compounds. This study investigated the effect of heat treatment on the enzyme activities and the antioxidant activities of soluble proteins from two Canadian lentil cultivars representing large green and small red seeds which were further separated into seed coat and cotyledons. Also, the effects of aqueous extracts of lentil on metmyoglobin reducing ability and inhibition of linoleic acid oxidation were tested. Large green lentil (*var.* CDC Greenland) showed higher lipoxygenase and peroxidase activities than small red lentil (*var.* CDC Maxim CL). Soluble components from the seed coat exhibited higher superoxide dismutase activity than from the cotyledon, but showed lower activities in lipoxygenase, peroxidase and glutathione reductase regardless of the varietal difference. Heat treatment reduced lipoxygenase, peroxidase and glutathione reductase activities significantly, but had no effect on superoxide dismutase. Seed coat (hull) of both varieties displayed higher antioxidant activity than cotyledon in DPPH and ABTS radical scavenging assays, ferric ion reducing assay and ferrous ion chelating assay of soluble proteins. Heat treatment caused a significant increase in the ferrous ion chelating ability of cotyledon flour that had negligible activity without heat treatment. This may be due to the high amount of protein-bound phenolic compounds present in the seed coat. Seed coat extracts either in 70% (v/v) ethanol or in water contained higher amount of phenolic compounds, condensed tannins and flavonoids than cotyledon extracts, and were able to reduce metmyoglobin to form oxymyoglobin and prevented

linoleic acid oxidation initiated by metmyoglobin. These results suggest that enzymes and antioxidant activities are distributed distinctly between lentil seed coat and cotyledon, and they are important in manipulating the quality of raw meat products. Pre-heat treatment in the form of radiation heating with infrared energy (up to 150 °C) or conduction heating in water bath (up to 90 °C) were effective in deactivating deleterious enzymes of the intact seeds to a great extent without altering the compounds with antioxidant activity and making lentil flour a suitable binder for raw meat products.

3.2 Introduction

Lentil (*Lens culinaris*) is an important legume crop that has been cultivated since 6800 BC (Sandhu & Singh, 2007) for human consumption. It is a good nutritional complement to cereals such as rice and wheat (Grusak, 2009). Lentil is considered as a good source of protein which provides both essential and non-essential amino acids, an excellent source of starch and also of dietary fibres (Faris et al., 2013) with a relatively low fat content (Candela et al., 1997). Based on its composition, lentil can be used as ground flour in various food systems, such as bread (Aider, Sirois-Gosselin & Boye, 2012), pasta (Wójtowicz & Mościcki, 2014) and snack food (Ryland, Vaisey-Genser, Arntfield, & Malcolmson, 2010). Previous studies from our group (Der, 2010; Pathiratne, 2014) utilized lentil flour as a gluten-free binder in fresh beef burgers that resulted in increased cooking yield without altering sensory properties.

Similar to many other legume seeds, heat treatment, mostly in the presence of excess water is necessary to make lentil palatable. However, when using legume flour as a dry ingredient, a precooking treatment shortens the total cooking time of the product as well as improves nutrient availability of legume products (Cenkowski & Sosulski, 1997). Among these pre-heat treatments, infrared (IR) heating, that involves utilizing electromagnetic radiation in the infrared region (1.8 - 3.4 µm) (Zheng et al., 1998), can decrease surface microbial load and activity of oxidative enzymes while improving protein digestibility, sugar availability, and palatability with increasing

soluble dietary fibre content (Satya et al., 2010). Der (2010) showed that the pre-cooked lentil flour when used as a binder in food systems such as fresh, ground beef products exerted beneficial effects by decelerating fresh red colour deterioration and lipid oxidation. Pathiratne, Shand, Pickard and Wanasundara (2015) also found that when heat is applied to reach lentil seed surface temperatures between 115 °C to 165 °C, deactivation of lipoxygenase and peroxidase may occur. Lipoxygenases catalyze the hydroperoxidation of polyunsaturated fatty acids such as linoleic acid with molecular oxygen (Khalyfa et al., 1990; Akyol et al., 2006). Peroxidases are enzymes that can catalyze the oxidation of a substrate and induce the reduction of peroxide species (Gijzen, van Huystee, & Buzzell, 1993). These two oxidative enzymes contribute to lipid oxidation and quality deterioration in either lentil seeds or in the products that contain lipids such as meat. Although a possible connection can be drawn with lipoxygenase inactivation due to heat treatment of lentil and prolongation of fresh red colour of beef from discoloration, it is not conclusive how this indirect effect can provide such a strong outcome in fresh, minced red-meat products. Moreover, pre-heat treatment enhances the value of lentil as a binder in fresh meat products in addition to its inherent gluten-free quality attribute. Scientific reasoning for this observation may substantiate the development of a lentil flour based gluten free binder for meat products.

There is accumulating evidence on various types of phytochemicals identified in lentil which include phenolic compounds, phytosterols, phytic acid, saponins, lectins, defensin and protease inhibitors (Faris et al., 2013). According to the work by Amarowicz and Pegg (2008), Xu and Chang (2007) and Zhang et al. (2015), the phenolic compounds are the main contributors to the antioxidant activity that alcoholic extracts of lentil exhibit in *in vitro* assays. Working with the seed coat and cotyledons that can be physically separated from lentil seed, Oomah et al. (2011) showed that the seed coat contains a high level of phenolic compounds compared to the cotyledon, therefore the seed coat may contribute a pool of more active molecules to the overall antioxidant activity of lentil whole seed extracts. However, this information does not directly

explain the situation reported by Der (2010) and Pathiratne (2014), because all the phenolic compounds that are soluble in organic solvents are not readily available to impart antioxidant activities when ground lentil seed is added to minced meat products. Water is the major available solvent in fresh ground meat products. Therefore, consideration of water soluble components may be reasonable to explore the beneficial activities that lentil flour imparts in fresh meat products. Tsai and She (2006) studied protein-bound phenolic compounds of pea and showed the significance of the protein-phenolic complexes in the antioxidative potential of the water extracts from the pea. These compounds could be the source of antioxidants in lentil as well. Also, little is known on the antioxidant activity changes after infrared heat treatment of lentil, because new antioxidants, such as Maillard reaction products could be produced, thus contributing to the overall antioxidant activity.

Seeds are known to contain a range of enzymes and among these, superoxide dismutase and glutathione reductase can protect plant cells from oxidative damage caused by reactive oxygen species under oxidative stress (Gill et al., 2013). It is our hypothesis that the mixture of phenolic compounds and enzymes which become soluble in the available water in a meat-lentil system represent the pool of antioxidant molecules available for the reaction cascade that led to the observed outcome by Der (2010) and Pathiratne (2014). In addition, the heat treatment of lentil that enhanced the red colour protection needs to be investigated in terms of its effect on the availability of water soluble compounds of lentil and also for their putative antioxidant activities.

Myoglobin is the major pigment of fresh meat and the oxidation of oxymyoglobin to metmyoglobin is the major reason for discoloration of raw meat and raw meat products (Mancini & Hunt, 2005). The addition of phenolic rich extracts, such as grape seed, rosemary and olive leaf into meat products were shown to be effective in protecting meat colour and in inhibiting lipid oxidation (Suman and Joseph, 2013). Rather than maintaining the form of oxymyoglobin, it was found that phenolic compounds were able to reduce metmyoglobin to form oxymyoglobin in a model system (Miura et al., 2014). This might be how the phenolic compounds present in lentil

react with metmyoglobin to slow down the discoloration of raw meat products. In addition, phenolic compounds act as antioxidants on unsaturated lipids by performing as free radical scavengers to eliminate reactive oxygen species and lipid peroxy radicals and as metal chelators to prevent initiating effects coming from free metal ions (Jakobek, 2015; Kanner, 1994). On one hand, phenolic compounds were found to scavenge peroxy radicals to inhibit oxidation of unsaturated fatty acids (Chimi, Cillard, Cillard, & Rahmani, 1991). On the other hand, common phenolic compounds such as catechin and quercetin showed antioxidant activity against the oxidation of linoleic acid induced by copper ion in a linoleic acid emulsion system (Beker, Bakır, Sönmezoğlu, İmer, & Apak, 2011). Other than metal ions, different molecular species of myoglobin and hemoglobin can also contribute to the oxidation of unsaturated fatty acids (Kühn et al., 1981). Fortunately, phenolic compounds also exhibit antioxidative effect against myoglobin coupled lipid oxidation systems. Utilizing red wine pigment mainly consisting of anthocyanins, Goupy et al. (2009) showed linoleic acid oxidation induced by metmyoglobin can be slowed down by 50% at 37 °C compared to the rate without phenolics. Similarly, phenolic compounds from lentil may also perform such effects to slow down the oxidation of linoleic acid. In this way, lipid oxidation occurring in raw meat products can be inhibited by lentil flour.

Accordingly, the objective of this study was to investigate the distribution of oxidative enzymes and other antioxidative components, mainly antioxidative enzymes and water soluble proteins (bound phenols), and phenolic compounds in the physical components (seed coat and cotyledon) of lentil and their ability to reduce metmyoglobin and to retard linoleic acid oxidation initiated by metmyoglobin in model systems. Assessment of heat treatments on the oxidant and antioxidant level of two widely cultivated lentil types in Canada was considered for this investigation.

3.3 Materials and Methods

3.3.1 Raw materials

For this study, two lentil cultivars representing popular market classes and widespread production; CDC Greenland (large green lentil) and CDC Maxim CL (small red lentil) that were grown in Saskatchewan, Canada. The seeds were harvested in 2013 and received in April and May, 2014, respectively. Incoming seeds were stored in plastic bags at room temperature prior to use.

Acetone, polyvinylpolypyrrolidone (PVPP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ethylenediaminetetraacetic acid (EDTA), 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p'*-disulfonic acid monosodium salt hydrate (FerroZineTM), ferrous chloride, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,4,6-tripyridyl-*s*-triazine (TPTZ), linoleic acid, TroloxTM, gallic acid, Folin-Ciocalteu reagent, potassium persulfate, NDAPH, methionine, glutathione disulfide, nitro blue tetrazolium, *o*-dianisidine, butanol, ferric ammonium sulfate, cyanidin, sodium nitrite, aluminum chloride, catechin and ascorbic acid used were all chemical analysis grade. Lipxygenase (from soybean), peroxidase (from horseradish), glutathione reductase (from baker's yeast), superoxide dismutase (from bovine erythrocyte) were purchased from Sigma Aldrich.

3.3.2 Lentil seed processing

3.3.2.1 Seed Tempering

Prior to heat treatment, approximately, 3 - 4 kg of whole lentil seeds for each treatment were tempered in polyethylene bags (30.48 cm × 40.64 cm) by adding a pre-determined amount of deionized water according to the AACC method 26-95.01 (AACC, 2000). The bags were heat sealed and then shaken manually for even distribution in the water. The seeds were allowed to temper at ambient temperature for 24 h to achieve equilibrium of 23% final moisture content. Three replications of tempering and heat treatment were carried out.

3.3.2.2 Heat treatment-Infrared (IR) heating

Seed heat treatment or “micronizing” was done at InfraReady Products (1998) Ltd. (Saskatoon, SK Canada). Tempered lentil seeds as described above were heat treated on a laboratory scale Micronizer (Model A, FMC Syntron Bulk Handling Equipment, Homer City, PA) composed of a propane heating element with two sets of three ceramic tiles (Model R, Rinnai, Japan), a Syntron feeder (Model F010, Riley Automation Ltd., Derby, England) and a Syntron magnetic feeder (Model BF2 A, FMC Corporation, Homer City, PA) similar to that was described by Pathiratne et al. (2015). The conveyor speed was controlled by the vibrating bed and it was adjusted to achieve a constant seed temperature prior to the final collection. A hand held IR Temp Gun thermometer (Oakton, Vernon Hills, IL) was used to measure the surface temperature of seeds coming out of the conveyor. All lentil seeds were treated to achieve seed surface temperature of 115 °C or 150 °C.

3.3.2.3 Heat treatment-Water bath heating

Approximately 70 g of tempered lentil seeds were weighed and transferred into polyethylene bags (20.32 cm × 25.40 cm) and vacuum sealed as a single seed layer. The water bath was filled with distilled water and heated to 90 °C. The bags were then kept in a water bath maintained at 90 °C and heated for 30 min. All the heat treatments were done in triplicate.

3.3.2.4 Seed component separation and grinding

Lentil seeds of both heat treatments were air dried under ambient temperature for 12 h on plastic trays to reach a moisture content below 12%. Seeds were separated into cotyledon and seed coat using an abrasive mill equipped with belt-drive blower (Dayton 6K778G, Niles, IL) and an aspirator (Crop Development Centre, University of Saskatchewan, Saskatoon, SK Canada). Contamination of samples was minimized by thoroughly cleaning the mill in between samples. Whole lentil seed, cotyledon and seed coat with or without heat treatment were then ground to obtain flours using a mill with an attachment that particles pass through 250 µm sieve (Ultra

Centrifugal Mill ZM 200, Retsch Co., Haan, Germany). All whole seed, seed coat and cotyledon flour samples were separately placed in plastic bags, stored at 4 °C and used for all analysis.

3.3.2.5 Compositional analysis

The moisture and ash content of lentil seed samples were measured according to the AOAC methods 925.10 and 923.03, respectively (AOAC, 1990). The crude fat and crude protein content were measured according to the AACC methods 30-25.01 and 46-11.02, respectively (AACC, 2000). Duplicate samples were analyzed.

3.3.2.6 Preparation of extracts for enzyme activities

Lentil flour samples were extracted with a potassium phosphate buffer (100 mmol/L, pH 7.6) for glutathione reductase and superoxide dismutase and with sodium phosphate buffer (50 mmol/L pH 6.9) for lipoxygenase and peroxidase activity assessment. Each flour sample was mixed with the respective buffer (1:10, w:v) using a magnetic stirrer in the cooler maintained at 4 °C for 2 h. The mixtures were then centrifuged at 10,000 x g for 30 min at 4 °C and the supernatant was saved as the crude enzyme extract. The protein content of these extracts was determined according to the method of Bradford (1976).

3.3.2.7 Preparation of soluble protein for antioxidant assays

Protein extracts were prepared according to the method of Arcan and Yemenicioğlu (2007). A dry flour sample of 7.5 g was homogenized for 1 min with 37.5 mL of cold acetone. The slurry was filtered under vacuum and the residue was collected and re-extracted. Homogenization and filtration of the residue was repeated twice and the residual defatted powder was air-dried overnight to remove excess acetone. The powder was then stored at -18 °C until further extraction. Acetone washed powder (~4.5 g), 0.1 g of insoluble PVPP (expected to bind phenolics) and 36 mL of deionized water was mixed, and extracted for 2 h at room temperature with a magnetic stirrer. The extract was filtered through 4 layers of cheesecloth to collect the filtrate. The filtrate was then centrifuged at 15,000 × g for 30 min at 4 °C and the supernatant was

dialyzed (Molecular weight cut-off: MWCO, 3500 Da) against deionized water for 72 h at 4 °C. After dialysis, the extract was centrifuged at 4500 x g for 15 min at 4 °C and the extract was freeze-dried and stored at -18 °C until later use. For antioxidant assays, 1 mg/mL of the freeze-dried powder suspension in deionized water was prepared and used.

3.3.2.8 Preparation of aqueous ethanolic and water extracts for myoglobin related assays

Flours of whole seed, seed coat or cotyledons (1.0 g) of small red lentil were separately placed in a 50 mL centrifuge tubes. For each tube, 10 mL of solvent which was either 70% (v/v) ethanol or deionized water (without any additive) was added, mixed and then vortexed for 30 s. The mixture was then shaken at 100 rpm for 12 h on an orbital shaker and at ambient temperature. The contents in the tubes were then centrifuged at $5,000 \times g$ for 10 min at 4 °C, and the supernatants were collected. The residues were re-extracted one more time using the same solvent at the same ratio. For each sample, the supernatants recovered from two extraction rounds were combined and filtered through a Whatman No.1 filter paper. The extracts were made up to 25 mL with the respective solvent and stored at -20 °C for later use.

3.3.3 Assays of enzyme activities

3.3.3.1 Superoxide dismutase activity

Superoxide dismutase activity was determined according to the method of Elavarthi and Martin (2010). The 2 mL assay substrate contained 50 mmol/L phosphate buffer (pH 7.8), 9.9 mmol/L L-methionine, 55 μ mol/L nitro blue tetrazolium (NBT), and 0.025% Triton-X 100 (w/v). A 40 μ L of phosphate buffer extract and 20 μ L 1 mmol/L of riboflavin were added to initiate the reaction. The reaction tubes containing extracts were illuminated under ambient light (~1000 lux) for 10 min while duplicate tubes with the same reaction mixture were covered with aluminum foils and kept in the dark cabinet and used as blanks. The superoxide radicals ($O_2^{\bullet-}$) generated due to photosensitization of riboflavin generates singlet oxygen and superoxide anion which cause conversion of NBT to NBT-diformazan and act as an indicator of $O_2^{\bullet-}$ production. The

absorbance of assay mixture was monitored as conversion of NBT to blue chromogen at 560 nm and the activity unit was calculated using a series of superoxide dismutase standards (0 - 650 unit/mL assay, 1 unit is defined as the inhibition of the rate of reduction of cytochrome C by 50% in a coupled system using xanthine and xanthine oxidase) (from bovine erythrocyte) of different concentrations.

3.3.3.2 Glutathione reductase activity

Glutathione reductase activity of phosphate buffer extracts was determined according to the method described by Rao, Paliyath, & Ormrod (1996). The assay mixture (1 mL) consisted of 100 mmol/L potassium phosphate buffer (pH 7.8), 2 mmol/L EDTA, 0.2 mmol/L NADPH, 0.5 mmol/L glutathione disulfide and 100 μ L of sample extract. The enzyme-catalyzed conversion of glutathione disulfide was initiated by adding NADPH at 25 °C. Reduction of glutathione disulfide to form reduced glutathione causes oxidation of NADPH which can be determined via measuring absorbance at 340 nm. The absorbance values of the samples were measured at 340 nm (extinction coefficient 6.2 mmol/L·cm). The activity unit was calculated from a series of glutathione reductase standard (0 - 50 unit/mg protein, 1 unit is defined as the reduction of 1 mmol of oxidized glutathione per minute) (from baker's yeast) of different concentrations.

3.3.3.3 Lipoxygenase activity

Lipoxygenase activity was measured according to the method of McCurdy, Nagel, & Swanson (1983). Briefly, 10 μ L of phosphate buffer extract was transferred to a quartz cuvette containing 3 mL of 0.946 mmol/L linoleic acid (LA) substrate (in pH 6.9 buffer containing 0.23 mmol/L Tween 20, 50 mmol/L phosphate and 50 mol/L borate), and mixed by inverting the cuvette. Lipoxygenase in the extract causes oxidation of LA and produces hydroperoxy lipid product containing conjugated dienes which strongly absorb at 234 nm. The absorbance of assay mixture was monitored at 234 nm using a Shimadzu UV-1800 spectrophotometer (Japan) after

mixing continuously for 5 min. One unit of lipoxygenase activity was calculated as the change of 0.001 Abs at 234 nm per minute from the slope of the linear region obtained.

3.3.3.4 Peroxidase activity

Peroxidase activity was determined according to the method of Chakraborty and Pradhan (2011). In brief, the substrate solution was prepared by mixing 2 mL of 50 mmol/L phosphate buffer (pH 6.9), 45 μ L of 30% hydrogen peroxide, and 100 μ L of 5 mg/mL *o*-dianisidine.

Peroxidase can catalyze the reduction of hydrogen peroxide and produce oxygen which reacts with *o*-dianisidine to form a red colour oxidized product and can be monitored at 460 nm.

Peroxidase activity determination was initiated by mixing 0.1 mL phosphate buffer extract with the substrate solution. Increase in absorbance at 460 nm was monitored and the slope of the linear region was obtained. A series of standards (0 - 50 units/mL assay, 1 unit is defined as oxidation of 1 mmol ABTS per minute) prepared using peroxidase from horseradish was used for calculating activity of the sample extracts.

3.3.4 Assays of antioxidant activity of soluble proteins (freeze dried powder)

3.3.4.1 Phenolic content

The phenolic content was determined according to the method of Asami, Hong, Barrett, and Mitchell (2003) and Singleton, Orthofer and Lamuela-Raventos (1999). In brief, 5 mL of deionized water, 0.5 mL of sample solution and 0.5 mL of Folin-Ciocalteu phenol reagent were added into 25 mL volumetric flask. After 5 min, 5 mL of 7% (w/v) Na_2CO_3 solution was added and the volume was made up to 25 mL using deionized water. The mixture was incubated for 2 h at ambient temperature and read for absorbance of blue chromogen at 765 nm against a blank solution without sample added. A standard curve was prepared using gallic acid (0 - 1.6 mg/mL). Phenolic content of the sample was expressed as milligrams of gallic acid equivalents per gram of freeze dried material.

3.3.4.2 DPPH radical scavenging ability

DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay was applied to the samples according to the method described by Brand-Williams, Cuvelier, & Berset (1995). Briefly, 0.1 mL of sample solution was added to 3.9 mL of 6×10^{-5} mol/L DPPH in methanol (purple colour). The decrease in absorbance at 515 nm due to reduced form of DPPH (colourless) was measured after 30 min. TroloxTM (0 - 1.6 μ mol/mL) was used to develop a standard curve. The free radical scavenging ability is expressed as equivalents of TroloxTM per gram of freeze dried powder.

3.3.4.3 ABTS radical scavenging ability

ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) assay of the samples was measured according to the method of Re et al. (1999). In brief, ABTS radical cations were prepared by mixing equal volumes of 7 mmol/L ABTS and 4.9 mmol/L potassium persulfate, and the solution was left to stand in the dark for 12 - 16 h at room temperature. Then the above solution was filtered and diluted with 80% (v/v) ethanol to have absorbance value of about 0.7 at 734 nm. For the assay, 0.2 mL of sample was added to 3.8 mL of ABTS solution. Due to antioxidant molecules in the sample, the blue chromogen (Abs 734 nm) ABTS radical cation converts to its neutral colourless form. Therefore, the absorbance of the assay mixtures was recorded at 734 nm after 30 min of incubation at ambient temperature. The ABTS assay results were expressed as micromoles of TroloxTM equivalents per gram of dry powder. TroloxTM (0 - 1.6 μ mol/mL) was used to develop the standard curve.

3.3.4.4 Ferric ion reducing antioxidant power (FRAP) assay

The FRAP assay described by Benzie and Strain (1996) and Pulido, Bravo and Saura-Calixto (2000) was used. Briefly, 900 μ L of FRAP reagent, prepared freshly and warmed to 37 °C, was mixed with 90 μ L of deionized water and 30 μ L of test sample or solvents for the reagent blank. The final dilution of the test sample in the reaction mixture was 1:34 (v:v). The FRAP reagent

contained 2.5 mL of a 10 mmol/L 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mmol/L HCl, 2.5 mL of 20 mmol/L ferric chloride and 25 mL of 0.3 mol/L acetate buffer (pH 3.6). Due to the reducing ability of antioxidant molecules in the sample, the ferric-TPTZ complex is reduced to ferrous form that has an intense blue colour and can be measured at 593 nm. The absorbance at 593 nm was recorded after exactly 4 min of mixing. Ferrous sulfate was used to build up a standard curve (0.0 - 3.0 mmol/L). The antioxidant ability is expressed as the equivalent of ferrous sulfate per gram of dry powder.

3.3.4.5 Ferrous ion chelating assay

Ferrous ion chelating assay that was described by Dinis, Madeira, & Almeida (1994) was utilized. In brief, 1 mL of sample (2 mg/mL) was mixed with 3.7 mL of 10% ammonium acetate buffer and 0.1 mL of 2 mmol/L ferrous chloride. In the reaction, the un-chelated FeCl_2 in the mixture was detected by adding 0.2 mL of 5 mmol/L FerroZineTM (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine). The absorbance of thoroughly mixed assay mixture was determined at 562 nm (Fe^{2+} -ferrozine complex, magenta colour) after 10 min of incubation at ambient temperature. EDTA at 2 mg/mL was used as positive control (100% chelation) and water with no chelator added was used as blank (0% chelation). The ferrous ion chelating activity of samples was expressed as percentage of inhibition of the ferrozine-Iron (II) complex formation.

3.3.5 Phenolic content of lentil extracts (70% v/v ethanol and water)

3.3.5.1 Total phenolic content (TPC)

Total phenolic content was determined using the same method as described in section 3.3.4.1. The total phenolic content was expressed as milligrams of gallic acid equivalents per gram of dry weight sample using a standard curve prepared with gallic acid (0.0 - 1.6 mg/mL). Samples with TPC concentration above this range were diluted using water or 70% (v/v) ethanol.

3.3.5.2 Condensed tannin content (CTC)

Condensed tannin content of the extracts was determined according to the method of Porter, Hrstich and Chan (1985) and expressed as cyanidin equivalents. In brief, 6.0 mL of acidified butanol (5% concentrated HCl in *n*-butanol, v/v) was added to a 15 mL centrifuge tube with 1 mL of sample extract, 0.2 mL of 2% (w/v) ferric ammonium sulfate in 2 mol/L HCl and the content was mixed by vortexing. Then the tube containing the assay mixture was transferred to a boiling water bath and kept for 50 min. The absorbance of the hydrolyzed tannins and ferric complex (magenta colour) was taken at 550 nm after cooling down to ambient temperature. The blank which did not contain sample extract was also included in the assay and cyanidin (0 - 20 µg/mL) was used to build the standard curve. The condensed tannin content of the extract was expressed as milligram cyanidin equivalent per gram dry weight of the sample. Samples with CTC level above the standard curve range were diluted using water or 70% (v/v) ethanol.

3.3.5.3 Total flavonoid content (TFC)

The total flavonoid content was determined according to the method described by Jia, Tang and Wu (1999). In short, 1 mL of sample extract, 4 mL of deionized water and 0.3 mL of 5% (w/v) sodium nitrite was added into a 10 mL volumetric flask and mixed. After 5 min, 0.3 mL of 10% (w/v) aluminum chloride was added and another 2 mL of 1 mol/L sodium hydroxide was added to the mixture after 6 min. After that the volume of the assay mixture was adjusted to 10 mL with deionized water and the absorbance of the flavonoid-aluminum stable complex of flavonoids (yellow colour) was measured at 510 nm. A blank assay mixture was prepared with deionized water in the place of sample extract. The total flavonoid content was expressed as the milligram catechin equivalent per gram of dry sample weight using a standard curve prepared with catechin (0.0 - 0.8 mg/mL) in 70% (v/v) ethanol.

3.3.6 Metmyoglobin reducing ability

The metmyoglobin reducing power assay was performed according to the method described by Allen and Cornford (2006). In a semi-micro cuvette containing 0.625 mL of 0.2 mmol/L metmyoglobin (from equine skeletal muscle, Sigma-Aldrich) in Tris buffer (100 mmol/L, pH 7.2), sample extracts were added at 94 μ L, 188 μ L and 375 μ L and water or 70% (v/v) ethanol was added to make up the total volume to 1.25 mL. Lipoyxygenase dissolved in deionized water was added at 4.78×10^6 units/mL into the 1.25 mL assay solution. Superoxide dismutase dissolved in deionized water was added at 4.66×10^3 units/mL. Catechin dissolved in 70% (v/v) ethanol was added at 0.21 mg/mL. Ascorbic acid dissolved in deionized water was added at 0.5 mg/mL. For the control group, 0.625 mL of the deionized water or 70% (v/v) ethanol was added. The cuvettes with assay solutions were covered with lids and stored in ambient temperature with exposure to ambient light. Each assay mixture was monitored for the absorbance values of metmyoglobin between 400 and 800 nm in 1 nm intervals against a blank made of the corresponding solvent. This scanning was done at 30s (right after mixing), 1 h, 2 h, 4 h, 8 h and 24 h after mixing metmyoglobin solution with samples. The metmyoglobin level was calculated according to the equations as follows (AMSA, 2012):

$$\%MetMb = \left(1.395 - \frac{A_{572} - A_{730}}{A_{525} - A_{730}} \right) \times 100$$

in which, %MetMb represents the percentage of metmyoglobin in the assay solution; A_{525} , A_{572} and A_{730} are absorbance values at 525, 572 and 730 nm.

3.3.7 Metmyoglobin induced linoleic acid oxidation assay

The oxidation of linoleic acid as induced by metmyoglobin was assessed as a model system to assay antioxidant potential of lentil extracts according to the method of Kühn et al. (1981). A 2.4 mL of 2.86 mmol/L linoleic acid (in pH 6.9 buffer containing 0.23 mmol/L Tween 20, 50 mmol/L phosphate and 50 mol/L borate) was added into macro quartz cuvette (4.5 mL). To start the oxidation of linoleic acid, 0.05 mL of 0.1 mmol/L metmyoglobin solution in 100 mmol/L Tris

buffer (pH 7.2) was added into the cuvette, and 0.05 mL of sample extracts or reference solution was added immediately. As reference samples, ascorbic acid (25 mg/mL) was assayed at 10 and 25 μ L and catechin (0.54 mg/mL) was assayed at 10, 25 and 50 μ L. The content in the cuvette was mixed well and the change of absorbance at 234 nm due to conjugated dienes formation was monitored for 15 min in 5 s intervals using a Shimadzu UV1800 spectrophotometer. The oxidation rate of linoleic acid initiated by metmyoglobin was calculated from the linear part of the curve developed for absorbance change at 234 nm. One unit of activity was expressed as the 0.001 absorbance change at 234 nm for 60 s.

3.3.8 Statistical analysis

The means and standard deviations of in total 24 treatments (in triplicates) (cultivar: 2 levels, heat treatment or raw: 4 levels, seed component: 3 levels) were calculated. The effects of lentil cultivar, heat treatment, seed component and their interactions on the results of composition, enzyme activities, antioxidant assays, phenolic contents were analyzed using three way factorial ANOVA in GLM procedure of the SAS 9.4 program (SAS Institute, USA). The effects of treatments, time and their interactions on the results of metmyoglobin reduction and linoleic acid oxidation were analyzed using two way ANOVA in GLM procedure. The level of significance was set at $P < 0.05$. The Tukey test procedure was used to do multiple comparisons between individual treatments. The effects of independent variables and their interactions on dependent variables are shown in section 9.1.

3.4 Results and Discussion

3.4.1 Lentil composition

Lentil seed coat and cotyledon comprised ~9% and ~90%, respectively of the whole seed weight of both large green and small red lentil. The levels of moisture and ash of raw seed did not show a significant difference ($P > 0.05$) between large green and small red cultivars or the seed coat and cotyledon components (Table 3.1).

Table 3.1 Composition of lentil seed components of two cultivars with or without heat treatment (Mean \pm SD)

Treatment ^{1,2}	Seed component	Moisture (%)	Ash (%) ³	Protein (%) ³	Fat (%) ³
<i>Large Green Lentil</i>					
Raw seed	Whole seed	9.4 \pm 1.0 ^{abc}	2.7 \pm 0.1 ^{efgh}	27.0 \pm 2.2 ^{abcd}	1.2 \pm 0.4 ^{ab}
	Seed cotyledon	9.8 \pm 1.1 ^{ab}	2.7 \pm 0.1 ^{fghij}	28.7 \pm 2.4 ^a	1.3 \pm 0.3 ^{ab}
	Seed coat	8.9 \pm 1.0 ^{abc}	2.9 \pm 0.1 ^{def}	10.5 \pm 0.9 ^e	0.6 \pm 0.1 ^{ab}
Water bath heating (90 °C for 30 min)	Whole seed	9.4 \pm 0.6 ^{abc}	2.8 \pm 0.0 ^{efg}	27.0 \pm 2.6 ^{abcd}	1.1 \pm 0.5 ^{ab}
	Seed cotyledon	7.7 \pm 0.9 ^{abc}	2.6 \pm 0.1 ^{fghijkl}	27.9 \pm 2.9 ^{abc}	1.4 \pm 0.7 ^{ab}
	Seed coat	8.1 \pm 1.0 ^{abc}	3.9 \pm 0.0 ^a	11.7 \pm 0.9 ^e	1.0 \pm 0.5 ^{ab}
Infrared heating (115 °C)	Whole seed	11.0 \pm 2.4 ^a	2.8 \pm 0.0 ^{efg}	27.0 \pm 2.7 ^{abcd}	1.5 \pm 0.6 ^{ab}
	Seed cotyledon	11.1 \pm 1.9 ^a	2.7 \pm 0.1 ^{efgh}	28.3 \pm 3.1 ^{ab}	1.6 \pm 0.7 ^{ab}
	Seed coat	9.4 \pm 1.7 ^{abc}	3.2 \pm 0.1 ^c	10.1 \pm 1.1 ^e	0.6 \pm 0.3 ^{ab}
Infrared heating (150 °C)	Whole seed	5.9 \pm 2.4 ^{bc}	2.8 \pm 0.0 ^{efg}	27.5 \pm 1.7 ^{abc}	1.7 \pm 0.7 ^a
	Seed cotyledon	5.4 \pm 2.6 ^{bc}	2.7 \pm 0.1 ^{efghi}	29.0 \pm 1.7 ^a	1.5 \pm 0.1 ^{ab}
	Seed coat	5.1 \pm 2.8 ^c	3.0 \pm 0.1 ^{cd}	10.5 \pm 1.0 ^e	0.7 \pm 0.3 ^{ab}
<i>Small Red Lentil</i>					
Raw seed	Whole seed	8.1 \pm 0.2 ^{abc}	2.5 \pm 0.1 ^{ijklm}	25.0 \pm 2.12 ^{cd}	1.2 \pm 0.7 ^{ab}
	Seed cotyledon	7.9 \pm 0.1 ^{abc}	2.5 \pm 0.0 ^{klm}	26.5 \pm 2.0 ^{abcd}	1.3 \pm 0.3 ^{ab}
	Seed coat	8.0 \pm 0.0 ^{abc}	2.7 \pm 0.1 ^{fghijk}	9.5 \pm 1.6 ^e	0.6 \pm 0.5 ^{ab}
Water bath heating (90 °C for 30 min)	Whole seed	9.1 \pm 0.4 ^{abc}	2.5 \pm 0.1 ^{ijklm}	24.3 \pm 1.7 ^d	1.1 \pm 0.4 ^{ab}
	Seed cotyledon	8.8 \pm 1.2 ^{abc}	2.4 \pm 0.1 ^m	25.7 \pm 1.9 ^{bcd}	1.5 \pm 0.8 ^{ab}
	Seed coat	8.4 \pm 1.2 ^{abc}	3.6 \pm 0.1 ^b	11.1 \pm 1.0 ^e	0.9 \pm 0.7 ^{ab}
Infrared heating (115 °C)	Whole seed	10.9 \pm 1.7 ^a	2.6 \pm 0.1 ^{ghijklm}	25.4 \pm 2.9 ^{bcd}	1.1 \pm 0.4 ^{ab}
	Seed cotyledon	11.0 \pm 1.7 ^a	2.4 \pm 0.1 ^{lm}	26.3 \pm 2.4 ^{abcd}	1.1 \pm 0.5 ^{ab}
	Seed coat	9.5 \pm 1.6 ^{abc}	3.1 \pm 0.1 ^c	9.6 \pm 1.0 ^e	0.6 \pm 0.3 ^{ab}
Infrared heating (150 °C)	Whole seed	5.8 \pm 3.6 ^{bc}	2.5 \pm 0.1 ^{hijklm}	25.7 \pm 1.7 ^{bcd}	1.3 \pm 0.2 ^{ab}
	Seed cotyledon	5.7 \pm 3.7 ^{bc}	2.4 \pm 0.0 ^m	26.6 \pm 1.8 ^{abcd}	1.3 \pm 0.2 ^{ab}
	Seed coat	5.6 \pm 3.0 ^{bc}	2.9 \pm 0.1 ^{de}	9.1 \pm 1.0 ^e	0.5 \pm 0.3 ^b

¹N=3.

²Means with different superscripts within each column are significantly different (P<0.05)

³On Dry weight basis

Protein and lipid content of whole seeds of the two cultivars were not different ($P>0.05$) but the seed coat of both cultivars contained lower amounts than cotyledons (Table 3.1), indicating cotyledons were the major source of protein and lipids. The levels of ash, protein and lipid of these two lentil cultivars were within the range of values reported in literature (Singh, Singh, & Sikka, 1968; Bhatta, 1988; Canadian Grain Commission, 2013).

Infrared heating to 150 °C caused lower residual moisture content in the whole seed, seed coat and cotyledon components ($P<0.05$) than other heat treatments. In the IR heat treatment, to achieve higher surface temperature of seeds, the residence time of treatment (150 °C: 1 min 14 s for small red and 1 min 46 s for large green lentil) was longer than at the lower temperature (115 °C: 30 s for small red and large green lentil), therefore removal of more moisture from seed could be expected. In the water bath heating, the lentil seeds were vacuum packaged in plastic bags, therefore any loss of water soluble components during thermal treatment was prevented. There was no major change occurring in protein, fat and ash contents of the whole seed, cotyledon and seed coat due to heat treatments.

3.4.2 Enzyme activities – antioxidative enzymes

The plant antioxidant defense system consists of enzymatic and non-enzymatic antioxidant components which are distributed in various cell organelles including chloroplast, mitochondria, peroxisomes and apoplast, and act in concert when needed (Mittler, Vanderauwera, Gollery, & Van Breusegem, 2004). In the cellular environment, the antioxidants help in maintaining steady-state concentration of reactive oxygen species to function as signaling molecules rather than imparting cytotoxic effects (Navrot et al., 2006). Glutathione reductase (GR) in particular, catalyzes conversion of oxidized glutathione back to the reduced form of glutathione, which are considered as the most abundant redox couple in plant cells (May et al., 1998; Noctor & Foyer, 1998). GR protect cells from oxidative damage from reactive oxygen species under high stress conditions, such as exposure to metal-metalloids, salinity conditions and drought stresses (Gill et

al., 2013). Superoxide dismutase (SOD) catalyze the dismutation of superoxide free radical ($O_2^{\bullet-}$) to molecular oxygen and H_2O_2 (Bowler et al., 1992). It also plays an important role in the plant defense system. The evaluation of activities of SOD and GR of lentil seed upon heat treatment is important to understand the contribution of heat-treated lentil flour to prevent lipid oxidation in aqueous food system, such as meat products. The results of enzyme activities of lentil are shown in Figure 3.1.

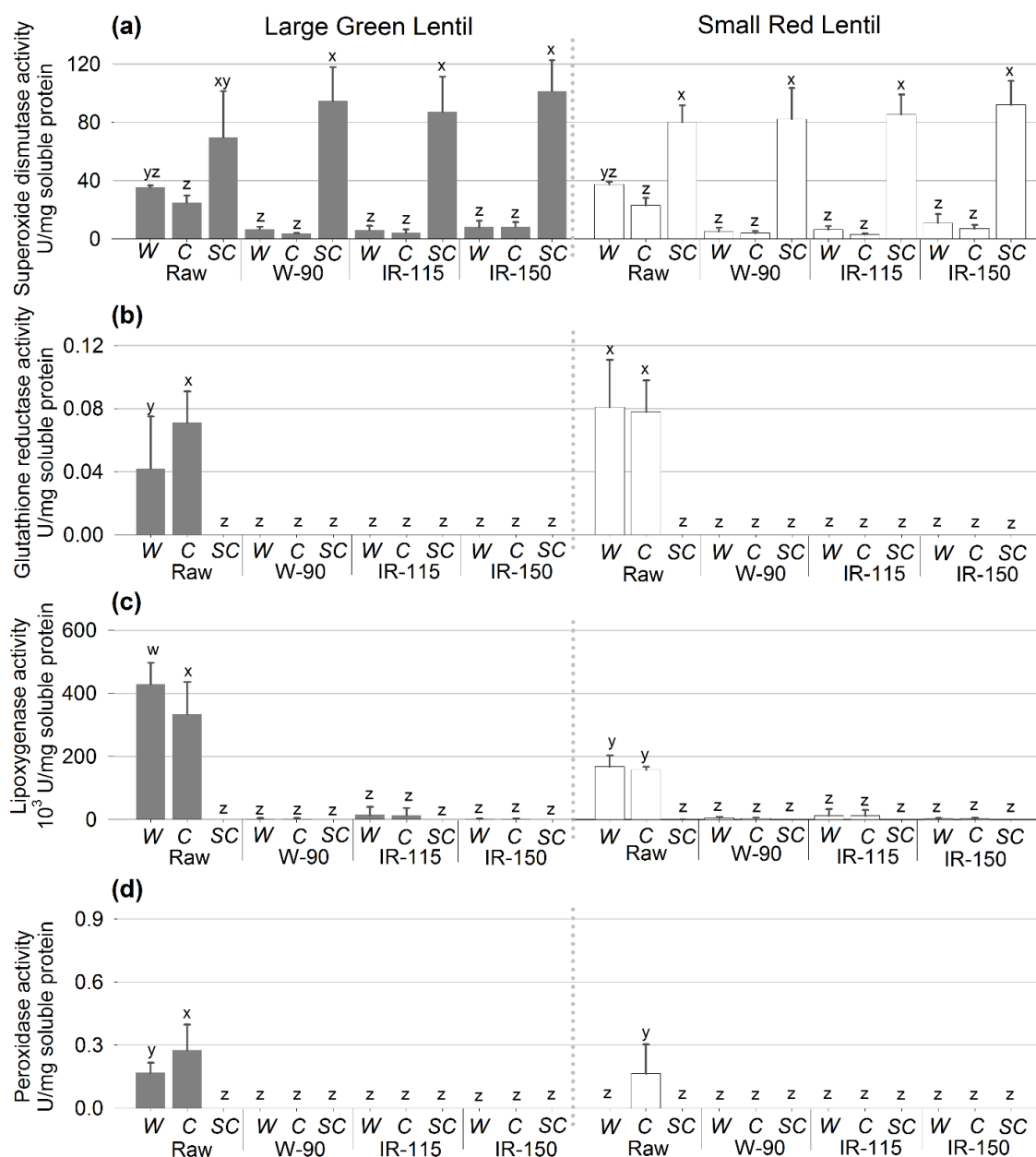


Figure 3.1 a) Superoxide dismutase activity, b) Glutathione reductase activity, c) Lipoxigenase activity and d) Peroxidase activity of lentil seed components of two cultivars with or without heat treatment. W: whole seed, C: cotyledon, SC: seed coat; Raw: raw lentil samples, W-90: water bath heated samples (90 °C for 30 min), IR-115: infrared heated samples to 115 °C, IR-150: infrared heated samples to 150 °C

Note: different letters (w, x, y and z) indicate significant difference ($P < 0.05$) between samples within a dependent variable.

3.4.2.1 Superoxide dismutase activity

Lentil cultivars did not differ ($P>0.05$) in superoxide dismutase activity (Figure 3.1a). When comparing seed components, the seed coat showed a higher ($P<0.05$) activity (ranging from 69.74 to 101.45 units/mg soluble protein) than that of the whole seed (5.20 to 37.46 units/ mg soluble protein) or cotyledon (3.02 to 24.75 units/mg soluble protein). After heat treatment of seeds, the SOD activity of whole seed, cotyledons and seed coat all showed no significant ($P>0.05$) change, but a decreasing trend was observed for SOD activity in whole seed and cotyledon. It is quite notable that the heat treatments did not show any differences ($P>0.05$) in the residual SOD activity observed of all treated samples. This suggests that the SOD present in these two cultivars of lentil are likely heat resistant. Superoxide dismutase activity was reported for legume seed coat, including lentil, faba bean and pea (Troszynska & Kubicka 2001). In their report, lentil seed coat showed the highest superoxide scavenging activity among these legumes. According to SDS-PAGE analysis, presence of superoxide dismutase has been confirmed in pea coat extracts. According to Tsai and She (2006) the SOD of pea is heat resistant and the phenolic compounds play a role in enhancing heat resistance and antioxidant capacity of pea SOD.

There are only a few reports on utilizing exogenous SOD in actual meat systems in order to enhance antioxidative status. In an *in vitro* study, SOD was found to be effective in inhibiting oxymyoglobin oxidation, but did not exhibit any effect on lipid oxidation (Gorelik and Kanner, 2001b). Also, together with ascorbic acid and catechin, SOD and other antioxidative enzymes can better perform as inhibitors of oxymyoglobin and lipid oxidation than acting alone in the model assay systems (Gorelik and Kanner, 2001b). It is reasonable to speculate that SOD in lentil can perform similar antioxidative effects in meat systems with the incorporation of lentil flour as a binder for fresh meat product. After heat treatment of lentil seeds, SOD may be the most active component in heat-treated lentil flour because SOD showed heat resistance and data of the present study supports this.

3.4.2.2 Glutathione reductase activity

The glutathione reductase (GR) or glutathione-disulfide reductase activity was found to be in the range of 0.04 to 0.08 units/mg protein extracted for the whole seed and seed cotyledon of both lentil cultivars (Figure 3.1b). The raw seed coat of both cultivars showed negligible activity (Figure 3.1b). The GR activity of whole seed of large green lentil was found to be lower than that of cotyledons, possibly due to a dilution effect with the presence of seed coat with no GR activity in the whole seed flour. Another explanation for this could be the phenolic compounds in the enzyme extracts of the whole seed that can inhibit the activity of glutathione reductase via forming complexes with protein, leading to precipitation and loss of function (Zhang, Yang, Tang, Wong, & Mack, 1997). The heat treatments resulted in complete abolishment of GR activity ($P < 0.05$) of cotyledons and the whole seed indicating thermal sensitivity of this enzyme. Also, the results indicated that GR activity of lentil can be inactivated with 30 min at 90 °C, which can be considered as the temperature that lentil may reach during home cooking for consumption. Glutathione reductase is present in leaf and root tissues of lentil plant as an antioxidative enzyme to catalyze the reduction of oxidized glutathione with NADPH (Bandoğlu, Eyidoğan, Yücel, & Avni Öktem, 2004). Under harsh circumstances, such as in salt stress, GR activity was increased in lentil leaf tissues in order to enhance the defensive ability against peroxides and reactive oxygen species (Bandoğlu et al., 2004). In seeds, glutathione reductase was also believed as part of the defense system and the activity increases with the imbibition and hydration of seeds (Cakmak, Strbac, & Marschner, 1993). There are few studies on the deactivation of GR upon heat treatment of lentil seeds or other species, but the activity of GR was found to be stable at temperature less than 45 °C (Mahan, Burke, & Orzech, 1990). However, from results of the present study, GR loses its activity after lentil was tempered to 23% moisture and heat treated at 90 °C, 115 °C or 150 °C.

3.4.3 Enzyme activities – oxidative enzymes

In contrast to those antioxidative enzymes, lipoxygenase (LOX) catalyzes the oxidation of *cis,cis*-1,4-pentadiene structure of unsaturated fatty acids, such as linoleic acid and linolenic acid (Khalyfa et al., 1990). Peroxidase (POX) can catalyze the oxidation of various types of substrate and induce the reduction of peroxide species (hydrogen peroxide or lipid peroxides) (Gijzen et al., 1993). Phenolic compounds are one type of substrate that peroxidase can catalyze (Caza, Bewtra, Biswas, & Taylor, 1999). Both of these enzymes are related to lipid oxidation and off-flavour production in seeds and vegetables (Lopez et al., 1994). The investigation of LOX and POX of lentil with or without heat treatment can give us an understanding on how heat treatments deactivate these oxidative enzymes present in raw lentil and help to prevent lipid oxidation in aqueous food systems.

3.4.3.1 Lipoxygenase activity

The activity of lipoxygenase (Figure 3.1c) of the raw seeds of two cultivars showed that the large green lentils exhibit higher ($P < 0.05$) activity than small red lentils prior to heat treatment. The lipoxygenase activities of whole seeds (416.91×10^3 units/mg protein for large green, 189.20×10^3 units/mg protein for small red lentil) was somewhat higher than those in cotyledons (278.34×10^3 units/mg protein for large green, 158.68×10^3 units/mg protein for small red). It is believed that heat created during abrasive milling of lentil may affect lipoxygenase activity, causing a lower activity of cotyledons than the whole seed (Savage, Wei, Sutherland, & Schmidt, 1995). Among the seed components, almost all the lipoxygenase activity was found in the cotyledon fraction (ranging from $157.45 - 334.49 \times 10^3$ units/mg protein) while the LOX activity in seed coat only ranged from $0.00 - 0.02 \times 10^3$ units/mg protein. Chang and McCurdy (1985) found that the lipoxygenase activity of lentil was 3720 units/mg flour (1 unit is defined as 0.001/min absorption change at 234 nm) and ranked as second highest activity next to soybean among the 14 legumes they studied. In comparison with the findings for lentil, soybean cotyledons are also

the major source of lipoxygenase activity, with over 350 times higher activity than that found in the seed coat (Žilić, Akıllıoğlu, Serpen, Perić, & Gökmen, 2013). The raw lentil seeds had higher ($P<0.05$) lipoxygenase activity compared with the heat-treated ones. This high lipoxygenase activity may result in seed rancidity during storage and could also induce lipid oxidation when incorporated into foods (Doehlert, Angelikousis, & Vick, 2010). Infrared heating to 150 °C and water bath heating at 90 °C for 30 min were able to reduce lipoxygenase activity ($P<0.05$) to a negligible level regardless of the cultivar or the seed component. It is well known that high temperature induces major structural changes in proteins (Ma et al., 2011), therefore structure related functions such as enzyme activities are decreased or abolished. Infrared heating of lentil to 115 °C surface temperature resulted in a decrease in the activity to $12.79 - 15.01 \times 10^3$ units/mg protein as shown in the present study. Infrared heating to 150 °C and water bath heating at 90 °C for 30 min caused a sharp reduction in the lipoxygenase activity to $1.91 - 5.06 \times 10^3$ units/mg (Figure 3.1c). Kermasha, Bisakowski, Ramaswamy and Van de Voort (1993) tested the effect of water bath heating and microwave heating at temperatures of 60, 70, 80 and 90 °C on soybean lipoxygenase activity and found that higher temperatures (80 and 90 °C) induced a faster decrease of activity than lower temperatures (60 and 70 °C). Brown, Wei, Steinberg and Villota (1982) did steam heating on soybean and found that lipoxygenase activity was almost deactivated at 91 °C. Although there are no studies on the optimum temperature for deactivating lipoxygenase of lentil, the results of the present study showed that lipoxygenase activity found in the lentil water soluble fraction was inhibited due to heating at 90 °C for 30 min.

3.4.3.2 Peroxidase activity

Peroxidase activity assessment of lentil seed (Figure 3.1d) showed that the activity was more concentrated in the cotyledon (0.09 to 0.22 units/mg soluble protein) than the whole seed (0.00 to 0.15 units/mg soluble protein) and seed coat (0.00 units/mg soluble protein). Basically, there was no activity detected in seed coat extracts. There was also no activity found in extracts of whole red lentil seed either. The reason could be that peroxidase is prone to have suicide inactivation in

the presence of phenolic compounds and hydrogen peroxide from attack by the radical intermediates (Baynton, Bewtra, Biswas, & Taylor, 1994). Among the cultivars studied, the raw seeds of large green lentil had higher peroxidase activity than the raw seeds of small red lentil ($P<0.05$). Interestingly, this observation is in contrast to the well-studied peroxidase distribution pattern in soybean which is also a legume. In soybean, according to peroxidase activity of seed coat, the soybean cultivars can be divided into two categories: low activity and high activity and this activity difference is regulated by a specific gene (Žilić et al., 2013; Gijzen et al., 1993). Heat treatment affected the activity of peroxidase in lentil cotyledons (Figure 3.1d) with complete inactivation. In green beans, heating caused a reduction in peroxidase and lipoxygenase activity, and with increase in the temperature of the treatment, there was low residual activity detected in the samples (Bahçeci et al., 2005).

Lipoxygenase and peroxidase may induce off-flavour or off-odour in seeds, because they catalyze the oxidation of unsaturated fatty acids (Burnette, 1977). There are few studies that have reported adding exogenous enzymes into meat systems to determine their effect on colour, oxymyoglobin oxidation or lipid oxidation. In the preliminary trials that were carried out in the present study, adding 10 mg of lipoxygenase (341.6×10^3 units/mg) or peroxidase (1301 units/mg) from commercial sources caused much faster browning of fresh ground beef compared to the control, suggesting that these two enzymes indeed can promote with the ongoing oxidation that occurs in fresh meat, consequently affecting the red colour.

3.4.4 Antioxidant activities of soluble proteins of lentil

The antioxidant activity of soluble proteins extracted from lentil was evaluated via four different antioxidant assays. These four assays tested radical scavenging ability, ferric ion reducing ability and ferrous ion chelating ability of soluble proteins extracted from different physical components of two cultivars with or without heat treatment.

3.4.4.1 Dry matter yield, protein content and phenolic content of water extracts

The dry matter recovered in the water extracts and the protein content of the recovered dry matter is given in Figure 3.2. Seed coat resulted in the lowest dry matter yield (0.6 - 1.3%) of the water extract and the level did not change significantly due to cultivar or heat treatment. Cotyledons gave a higher yield of dry matter than seed coat ($P < 0.05$), from 3.0% to 5.2% and the heat treatment significantly decreased ($P < 0.05$) the dry matter yield. Heat treatment such as boiling can cause a loss of solubility of lentil protein from over 50% to less than 25% (Ma et al., 2011). The total amount of protein and phenolic content of all samples account for 25.41 to 70.63% of the dry matter. Other than protein and phenolics, there could be some other compounds with high molecular weight, such as soluble fibre and starch.

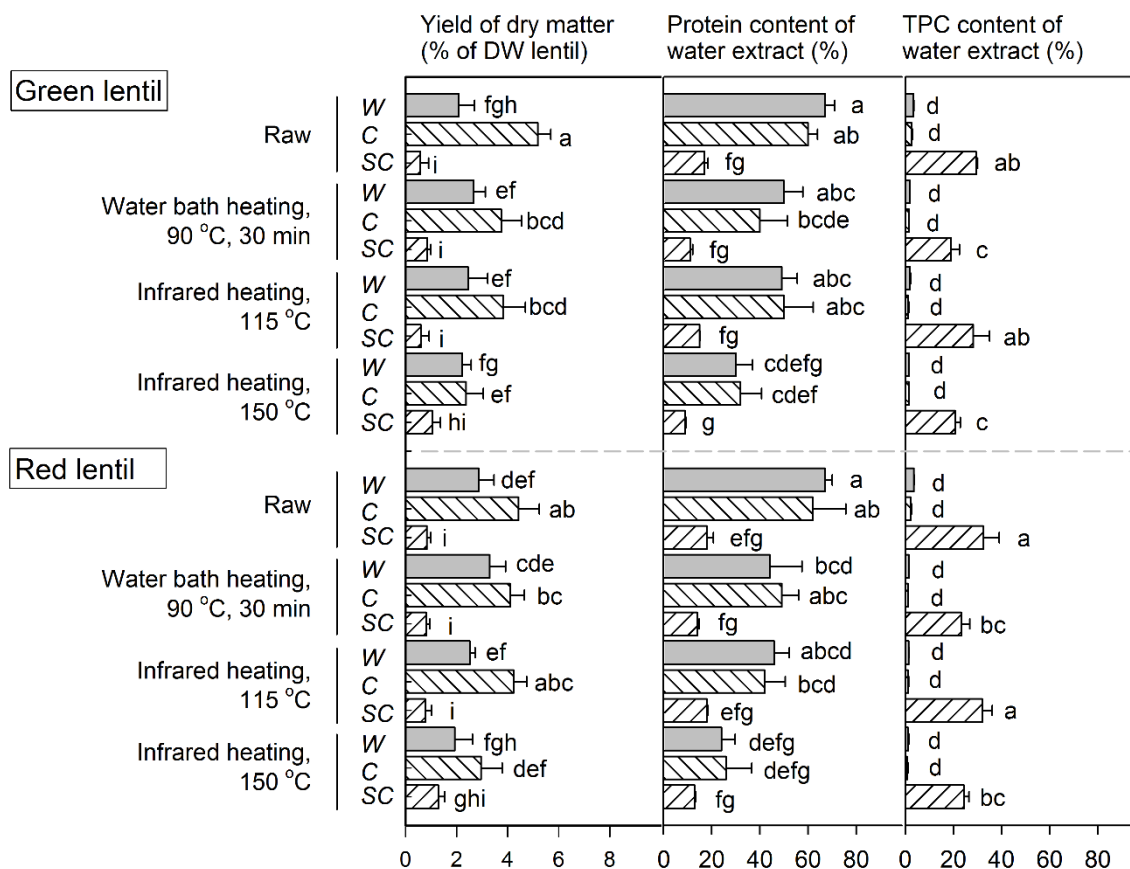


Figure 3.2 Dry matter yield of water extract (% of lentil dry weight), protein content of the water extract (%) and total phenolic content (TPC) of water extract (%) of lentil. W: whole seed, C: cotyledon, SC: seed coat

Lentil protein is comprised of water soluble albumins (16.8%), salt soluble globulins (49.0%), acid or alkaline soluble glutelins (11.2%) and alcohol soluble prolamins (3.5%) and the levels of these proteins could vary due to different extraction methods (Boye et al., 2010; Boye, Zare, & Pletch, 2010). In this study, since water was the only solvent, the major protein type here likely was albumins. Similar to the dry matter yield of water extracts, the level of proteins found in the dry matter was lower ($P < 0.05$) in seed coat samples than in the cotyledons or the whole seed samples. This is probably due to the naturally low total and soluble protein content in the seed coat compared to cotyledons as shown in Table 3.1. When the protein content of dry matter recovered from cotyledons and whole seeds was considered, no significant differences ($P > 0.05$)

in the protein content were found between raw and water bath heat-treated samples. Also, IR heat treatment to 115 °C did not cause a difference in protein content as compared to raw samples, but IR heated cotyledons and the whole seed had significantly lower ($P<0.05$) protein content than raw samples. According to Pathiratne et al. (2015), a higher temperature of infrared heating employed for precooking of lentil caused a decrease in protein dispersibility index (PDI) of the flour indicating that the level of protein solubility decreased.

Phenolic compounds including those found in seeds are capable of scavenging free radicals and chelating metal ions (El Gharras, 2009). The phenolic content determined for the water extracts of samples (Figure 3.2) indicate their availability to exert antioxidant activities. Not surprisingly, all seed coat groups contained higher ($P<0.05$) phenolics ranging from 19.1% to 32.1% of the extracts than the PC of whole seed or seed cotyledons (ranging from 1.1% to 3.6% of the extracts). None of the heat treatments had an effect on the PC of water soluble fractions of seed coat, cotyledon or whole seeds ($P>0.05$). The use of acetone and PVPP in the preparation of water extracts do not allow free phenolic compounds to be extracted. Therefore, only protein-bound phenolics were expected in the extracts upon recovery of soluble proteins. According to the recent review on protein-phenolic interactions (Ozdal, Capanoglu, & Altay, 2013), proteins and phenolic compounds may interact via hydrogen bonding, hydrophobic bonding and van der Waals forces which are considered as reversible interactions or via covalent bonds which are seen as irreversible. Aydemir and Yemenicioglu (2013) reported that proteins extracted from lentil also have bound phenolic compounds in high amounts and consequently exhibit antioxidant activity. The study by Bartolomé, Estrella and Hernández (2000) showed that phenolic compounds extracted from lentil can bind bovine serum albumin (BSA) protein to form a phenol-protein complex. Although these authors did not report any antioxidant activity, it provides evidence that proteins in lentil can exist in the form with bound phenolics. Kosińska, Karamać, Penkacik, Urbalewicz, and Amarowicz (2011) used tannins and storage proteins both extracted from broad bean seeds to test the interactions between phenolics and proteins and found that both soluble and

insoluble tannin-protein complexes can be formed. Because of the high level of phenolic compounds found in the seed coat water extracts, it will be interesting to see the antioxidant potential of the soluble proteins of the same lentil samples.

Phenolics can exert antioxidant activity by acting as chain-breakers. The mechanism of their activities are scavenging free radicals either by single electron transfer (SET) or by hydrogen atom transfer (HAT), acting as a reductant and donating electron, or forming chelates with transition metal ions (Fe^{2+} , Cu^{2+}) that promote oxidation (Prior, Wu, & Schaich, 2005). Therefore, an assay was conducted to understand what direction that the lentil soluble components interfere in lipid oxidation reactions of the biological systems.

3.4.4.2 DPPH radical scavenging ability

The results of antioxidant assays on soluble proteins extracted from large green and small red lentil are shown in Table 3.2 and Table 3.3, respectively. The DPPH assay measures the ability of antioxidants to reduce the stable radical DPPH to form the non-radical form DPPH-H in the reaction (Gülçin, 2012). There was no significant difference ($P>0.05$) observed between the two cultivars for DPPH radical scavenging ability of the extracts of whole seed, cotyledon or seed coat (Table 3.2 and Table 3.3). Han and Baik (2008) compared two cultivars of lentil (Pardina and Crimson) and found no significant difference in the DPPH radical scavenging ability of 80% ethanol extracts of the two cultivars. But Zhang et al. (2015) found that the total phenolic content, phytochemical composition and the DPPH radical scavenging ability differed among the 20 new lentil cultivars from Canada. Such variability may come from the genetic diversity of these different lentil cultivars.

Table 3.2 Antioxidant activities of water soluble compounds of different seed components of large green lentil with or without heat treatment (Means \pm SD)

Seed component	Treatment ^{1,2}	DPPH ³ (μ mol Trolox equivalent/g DW lentil)	ABTS (μ mol Trolox equivalent/g DW lentil)	FRAP ($\times 10^{-3}$ μ mol FeSO ₄ /g DW lentil)	Ferrous ion chelating (%)
<i>Large Green Lentil</i>					
Whole seed	Raw seed	0.27 \pm 0.28 ^c	0.43 \pm 0.40 ^e	2.10 \pm 0.72 ^c	0.00 \pm 0.00 ^f
	Water bath heating (90 °C, 30 min)	0.16 \pm 0.28 ^c	0.44 \pm 0.34 ^e	2.85 \pm 1.85 ^c	44.64 \pm 4.32 ^{cde}
	Infrared heating (115 °C)	0.06 \pm 0.05 ^c	0.63 \pm 0.55 ^e	2.63 \pm 2.10 ^c	37.46 \pm 14.92 ^{de}
	Infrared heating (150 °C)	0.17 \pm 0.24 ^c	0.73 \pm 0.38 ^e	2.49 \pm 1.67 ^c	39.49 \pm 14.86 ^{de}
Seed cotyledon	Raw seed	0.00 \pm 0.00 ^c	0.00 \pm 0.00 ^e	3.21 \pm 1.73 ^c	0.00 \pm 0.00 ^f
	Water bath heating (90 °C, 30 min)	0.33 \pm 0.57 ^c	0.87 \pm 0.28 ^e	3.12 \pm 2.73 ^c	26.63 \pm 5.32 ^e
	Infrared heating (115 °C)	0.19 \pm 0.32 ^c	0.76 \pm 0.54 ^e	2.93 \pm 2.69 ^c	36.47 \pm 17.68 ^{de}
	Infrared heating (150 °C)	0.14 \pm 0.25 ^c	0.45 \pm 0.06 ^e	1.15 \pm 0.49 ^c	38.35 \pm 14.47 ^{de}
Seed coat	Raw seed	6.90 \pm 3.48 ^b	2.96 \pm 1.62 ^{cd}	19.29 \pm 10.81 ^b	75.40 \pm 1.05 ^{ab}
	Water bath heating (90 °C, 30 min)	9.08 \pm 1.27 ^{ab}	4.16 \pm 0.82 ^{abc}	20.06 \pm 1.29 ^b	81.21 \pm 0.32 ^a
	Infrared heating (115 °C)	7.04 \pm 3.91 ^b	3.01 \pm 1.73 ^{bcd}	18.79 \pm 9.83 ^b	76.07 \pm 1.21 ^{ab}
	Infrared heating (150 °C)	11.38 \pm 3.51 ^{ab}	5.01 \pm 1.67 ^{ab}	25.46 \pm 7.57 ^{ab}	80.75 \pm 0.78 ^a
Catechin (0.2 mg/mL)		1.45 \pm 0.01	1.13 \pm 0.03	1.31 \pm 0.01	27.24 \pm 0.62

¹N=3

²Means with different superscripts within each column are significantly different (P<0.05)

³DPPH: 1,1-diphenyl-2-picryl-hydrazyl, ABTS: 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), FRAP: ferric reducing antioxidant potential

Table 3.3 Antioxidant activities of water soluble compounds of different seed components of small red lentil with or without heat treatment (Means \pm SD)

Seed component	Treatment ^{1,2}	DPPH ³ (μ mol Trolox equivalent/g DW lentil)	ABTS (μ mol Trolox equivalent/g DW lentil)	FRAP ($\times 10^{-3}$ μ mol FeSO ₄ /g DW lentil)	Ferrous ion chelating (%)
<i>Small Red Lentil</i>					
Whole seed	Raw seed	0.42 \pm 0.69 ^c	0.90 \pm 0.53 ^e	3.80 \pm 1.46 ^c	0.00 \pm 0.00 ^f
	Water bath heating (90 °C, 30 min)	0.32 \pm 0.55 ^c	0.33 \pm 0.27 ^e	2.63 \pm 0.93 ^c	44.04 \pm 10.04 ^{de}
	Infrared heating (115 °C)	0.50 \pm 0.69 ^c	0.78 \pm 0.01 ^e	2.97 \pm 1.69 ^c	35.23 \pm 14.91 ^{de}
	Infrared heating (150 °C)	0.12 \pm 0.21 ^c	0.44 \pm 0.15 ^e	1.22 \pm 0.52 ^c	53.60 \pm 17.77 ^{bcd}
Seed cotyledon	Raw seed	0.14 \pm 0.24 ^c	0.12 \pm 0.15 ^e	1.96 \pm 1.12 ^c	0.00 \pm 0.00 ^f
	Water bath heating (90 °C, 30 min)	0.40 \pm 0.69 ^c	1.02 \pm 0.21 ^{de}	3.37 \pm 3.44 ^c	31.53 \pm 3.31 ^{de}
	Infrared heating (115 °C)	0.58 \pm 1.00 ^c	1.24 \pm 0.16 ^{de}	3.86 \pm 4.15 ^c	25.79 \pm 13.25 ^e
	Infrared heating (150 °C)	0.14 \pm 0.23 ^c	0.64 \pm 0.18 ^e	1.67 \pm 0.83 ^c	33.87 \pm 16.03 ^{de}
Seed coat	Raw seed	9.54 \pm 1.59 ^{ab}	4.09 \pm 0.87 ^{abc}	30.17 \pm 10.86 ^{ab}	69.97 \pm 2.41 ^{abc}
	Water bath heating (90 °C, 30 min)	9.02 \pm 1.49 ^{ab}	4.05 \pm 0.79 ^{abc}	22.93 \pm 2.07 ^b	79.07 \pm 0.83 ^{ab}
	Infrared heating (115 °C)	9.19 \pm 2.84 ^{ab}	3.87 \pm 1.36 ^{bc}	26.87 \pm 8.45 ^{ab}	70.11 \pm 3.34 ^{abc}
	Infrared heating (150 °C)	13.68 \pm 1.68 ^a	6.07 \pm 1.23 ^a	37.10 \pm 7.87 ^a	75.57 \pm 1.11 ^{ab}
Catechin (0.2 mg/mL)		1.45 \pm 0.01	1.13 \pm 0.03	1.31 \pm 0.01	27.24 \pm 0.62

¹N=3

²Means with different superscripts within each column are significantly different (P<0.05)

³DPPH: 1,1-diphenyl-2-picryl-hydrazyl, ABTS: 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), FRAP: ferric reducing antioxidant potential

The DPPH radical scavenging activity of seed coat groups ranged from 6.90 to 13.68 μmol Trolox/g DW sample which was higher ($P < 0.01$) than the activities of seed cotyledon and whole seed ranged from 0.00 to 0.58 μmol Trolox/g DW sample. Seed coat extracts (0.191 - 0.326 mg/mL TPC) of both cultivars had higher DPPH radical scavenging ability than catechin at 0.2 mg/mL, suggesting a strong antioxidant ability of lentil seed coat. Dueñas, Hernández and Estrella (2006) have reported that seed coat exhibited higher DPPH radical scavenging ability than the cotyledons, which may have a direct relationship with the high content of phenolic compounds in the seed coat as pointed by Oomah et al. (2011). Heat treatment did not have a significant effect ($P > 0.05$) on the DPPH radical scavenging activity of the extracts of either cotyledon or seed coat. The results of DPPH radical scavenging activity suggest that the extracts of lentil soluble protein may also pose the ability to scavenge peroxy radicals that are produced by lipid oxidation in raw meat products to slow down such oxidation reactions.

3.4.4.3 ABTS radical scavenging ability

The ABTS assay measures the ability of the test component to react directly with the ABTS radical cation (Gülçin, 2012) and the reaction occurs in the aqueous environment. Similar to DPPH assay, this assay also uses stable-free radicals rather than radicals generated from oxidizing the substrate, therefore providing information more related to radical scavenging activity of the water extracts of lentil components.

The seed cultivar has a significant effect ($P < 0.05$) on the ABTS radical scavenging activity and small red lentil showed higher activity than the large green ones (Table 3.2 and Table 3.3). The ABTS radical scavenging showed a marked difference ($P < 0.05$) in the activity between the seed coat and cotyledon extracts of both cultivars (Table 3.2 and Table 3.3). As a general trend for extracts of large green lentil, the heat treatments actually caused an increase in the ABTS scavenging activity of the compounds extracted from seed coat, but not from cotyledon or whole seed. Arcan and Yemenicioğlu (2007) found that thermal treatment caused a significant ($P < 0.05$)

increase in the antioxidant activity determined by ABTS assay of soluble proteins extracted from chickpea and white bean. In another study by Fernandez-Orozco et al. (2003), the ABTS radical scavenging activity of lentil before and after cooking were tested and no difference was found in the activity due to the heat treatment. Žilić et al. (2012) found that the antioxidant activity of pea protein isolate as measured by ABTS scavenging assay increased after heat treatment. Xu and Chang (2008) suggested that thermal treatment of legume seeds may contribute to the release of protein-bound phenolic compounds by breaking down cellular constituents and thereby promoting higher antioxidant capability. In the present study, the general trend of radical scavenging ability exhibited by water soluble proteins of whole seed did not change due to heat treatment, therefore it can be assumed that water soluble components released by lentil flour in aqueous food environment maintains free radical scavenging ability even after receiving heat treatment as an ingredient pre-treatment. The ABTS scavenging ability of catechin at 0.2 mg/mL was 1.13 μmol Trolox equivalent/g DW lentil. It is worth noting that the soluble proteins of seed coat from red lentil after infrared heating to 115 °C showed higher ABTS radical scavenging ability than catechin (0.2 mg/mL), suggesting a beneficial effect of heat treatment.

3.4.4.4 FRAP

The FRAP assay mainly evaluates reducing ability of ferric ions of water soluble extracts of lentil. Ferrous (Fe^{2+}) and ferric (Fe^{3+}) ions can catalyze the formation of reactive oxygen species (ROS) and can boost lipid peroxidation via Fenton reaction (Stohs & Bagchi, 1995). In fact, both Fe^{3+} and Fe^{2+} are needed to initiate lipid peroxidation (Braugher, Duncan, & Chase, 1986). Therefore, the reduction of Fe^{3+} to Fe^{2+} is important for inhibiting lipid oxidation in aqueous food systems. In addition, Fe^{2+} and Fe^{3+} have been found to accelerate the oxidation of oxymyoglobin and to induce discoloration of raw meat products (Gorelik & Kanner, 2001a; Allen & Cornforth, 2006). The ability to reduce ferric ions would be a beneficial functionality when adding lentil flour into meat products as it would consequently prevent oxidation of both lipids and oxymyoglobin.

According to the data reported in Table 3.2 and Table 3.3, ferric reducing ability of the water soluble fractions recovered from the cotyledons, seed coat and whole seeds showed higher ($P < 0.05$) values for small red lentil than large green lentil. Zhang et al. (2015) investigated FRAP of 70% (v/v) acidic methanol extracts of various cultivars of Canadian lentils and did not find any difference between the cultivars of Maxim (Small red) and Greenland (Large green). This is probably because they only considered the extracts of whole seed rather than separately looking at cotyledon and seed coat. Among the seed components, seed coat of both cultivars ($18.79 - 37.10 \times 10^{-3} \mu\text{mol FeSO}_4/\text{g DW}$) showed higher ($P < 0.01$) FRAP than cotyledons ($1.15 - 3.86 \times 10^{-3} \mu\text{mol FeSO}_4/\text{g DW}$). Soluble proteins of whole seed showed a similar value of FRAP ($1.22 - 3.80 \times 10^{-3} \mu\text{mol FeSO}_4/\text{g DW}$) as cotyledon, rather than as seed coat, possibly because cotyledon accounts for the majority (~90%) of the seed weight and the activity of seed coat is diluted. Heat treatments, including infrared heating up to 115 and 150 °C and water bath heating of 90 °C had no effect on the FRAP values ($P > 0.05$) of whole seeds of both lentil cultivars. However, Xu and Chang (2009) reported a decrease (58.6 - 69.2%) in FRAP values of 70% acidic acetone extracts of lentil seed after various heat treatments. In the present study, some of the standard deviations are higher than others due to the inevitable mass loss during sample preparation, such as extraction, concentration and freeze drying. Most of the samples showed higher FRAP activity than catechin at 0.2 mg/mL, suggesting a very strong reducing ability from the seed coat and moderate contributions from the cotyledon and whole seed.

The FRAP assay measures the antioxidant power of the test material to reduce the ferric ion complex to the ferrous ion complex (Benzie & Strain, 1996). The ferric ion was found to generate more oxygen radicals in a liposome model and lipid peroxidation needs both ferric and ferrous ions (Stohs & Bagchi 1995). In the meat system, browning or discoloration is mainly due to the formation of the ferric form of the iron ion coordinated at the centre of metmyoglobin (Suman and Joseph, 2013). Since ferric ion in FRAP assay is also coordinated at the centre of TPTZ

(Benzie & Strain, 1996), the results of FRAP assay could be very helpful to anticipate the reducing effect on metmyoglobin.

3.4.4.5 Ferrous ion chelating ability

For the ferrous ion chelating assay (shown in Table 3.2 and Table 3.3), there is no significant difference between the activities observed for large green and small red lentils ($P>0.05$). For water soluble fractions of seed components, the ferrous ion chelating ability of seed coat (69.97 - 81.21%) is higher than that of cotyledons (0.00 - 38.35%) when they are at the same level (Table 3.2 and Table 3.3). The nil values from samples of raw cotyledons suggest that they do not pose ferrous ion chelating ability. The nil values observed for whole seed may indicate the dilution of components that are soluble from seed coat because the soluble components of seed coat (0.6 - 1.3% of DW lentil flour) were much lower than that of cotyledons (2.4 - 5.2% of DW lentil flour). It is interesting to note that heat treatment of whole seed and cotyledon actually caused a release of compounds that can increase the ferrous ion chelating ability ($P<0.05$), and this was observed for all the samples upon heat treatments regardless of how it was carried out (Table 3.2 and Table 3.3). Heat treatment did not cause a significant increase ($P>0.05$) in the chelating capacity of seed coat. In a previous study, thermal treatment caused an increase in the ferrous ion chelating capacity of soluble proteins extracted from white bean and chickpea (Arcan & Yemenicioğlu 2007), demonstrated that the soluble proteins after heat treatment of the legumes retained metal ion chelating capacity. This was probably because the heat treatment changed the structure of protein and more Fe^{2+} ion binding sites were exposed. Ueno, Urazono, and Kobayashi (2014) used bovine serum albumin (BSA) to form an iron-binding complex and found that increases in heating temperature increases led to less soluble iron, and that more iron was bound to proteins. The ferrous iron chelating ability of catechin at 0.2 mg/mL was 27.24%. But most of the cotyledon and whole seed samples showed higher ferrous iron chelating ability than catechin due to heat treatments.

The importance of ferrous ion chelating ability is related to the reduction of overall oxidative stress of the system by divalent metal ions because ferrous ions can be initiators of reactions leading to free radical generation (Choe and Min, 2009). Also, the ferrous ion is capable in catalyzing the conversion of hydrogen peroxide to hydroxyl radical via Fenton reaction, initiating lipid peroxidation (Stohs & Bagchi 1995). In addition, the ferrous ion is believed to catalyze the oxidation of oxymyoglobin, which causes discoloration of fresh meat (Gorelik and Kanner 2001a). Soluble proteins together with other phytochemicals can chelate these ions and inhibit the occurrence of free radicals (Elias et al., 2008). In the present study, the water soluble fraction of lentil seed coat showed ferrous ion chelating ability which could contribute to their possible antioxidant activity, and was much stronger compared to extracts of cotyledons or whole flour. Since heat treatment showed a promoting effect on ferrous ion chelating ability of the water extracts, it can be generally considered that such treatments are beneficial to the antioxidant ability of lentil seed and its components in aqueous environments.

3.4.5 Contents of total phenolics, condensed tannins, and total flavonoids of lentil extracts using water and 70% (v/v) ethanol

Since no significant difference was found in the antioxidant activity of water soluble extracts between large green and small red lentil, as well as enzyme activities except lipooxygenase, small red lentil was selected for further analysis. Also, the three types of heat treatments showed little difference on either antioxidant assays or enzyme activities of lentils, therefore the infrared heating to 150 °C was selected as the heat treatment to evaluate in the following analysis. In order to compare with many of the literature reports, both water and 70% (v/v) ethanol were separately employed as solvents to extract antioxidative components from whole seed, cotyledon and seed coat of lentil. Water extraction was preferred as it relates to what actually happens in meat or other food systems that lentil flour is added. Ethanolic extraction, on the other hand, was carried out to compare with the water extracts, since aqueous ethanol has been the solvent widely used in studies on phenolic compounds in lentil. The results are shown in Table 3.4.

Table 3.4 Total phenolic content (TPC), condensed tannins content (CTC) and total flavonoid content (TFC) of aqueous and ethanolic extracts of whole seed, seed coat and cotyledon of small red lentil (*var.* CDC Maxim CL) with or without infrared heat treatment (Means \pm SD)

Treatment ^{1,2}	Component	TPC		CTC		TFC	
		mg/g DW GAE ³	Contribution to Seed TPC ⁴	mg/g DW CE	Contribution to seed CTC ³	µg/g DW CE ³	Contribution to seed TFC
70% v/v ethanol							
Raw	Whole seed	1.40 ± 0.11 ^{de}		0.65 ± 0.04 ^d		0.53 ± 0.02 ^c	
	Cotyledon	0.44 ± 0.14 ^e	10.3%	0.07 ± 0.01 ^d	1.6%	0.27 ± 0.04 ^c	19.9%
	Seed coat	38.21 ± 1.12 ^a	89.7%	43.54 ± 1.07 ^a	98.4%	10.90 ± 0.41 ^a	80.1%
Heat-treated ⁵	Whole seed	1.22 ± 0.06 ^{de}		0.51 ± 0.03 ^d		0.49 ± 0.05 ^c	
	Cotyledon	0.52 ± 0.22 ^e	13.5%	0.08 ± 0.01 ^d	2.1%	0.27 ± 0.03 ^c	20.5%
	Seed coat	33.24 ± 1.68 ^b	86.5%	37.57 ± 0.79 ^b	97.9%	10.49 ± 0.20 ^a	79.5%
Water							
Raw	Whole seed	4.21 ± 0.57 ^d		0.33 ± 0.06 ^d		0.36 ± 0.11 ^c	
	Cotyledon	4.26 ± 0.21 ^d	62.3%	0.22 ± 0.07 ^d	21.3%	0.14 ± 0.01 ^c	14.2%
	Seed coat	25.76 ± 2.79 ^c	37.7%	8.12 ± 1.53 ^c	78.7%	8.46 ± 1.07 ^b	85.8%
Heat-treated	Whole seed	1.98 ± 0.14 ^{de}		0.24 ± 0.01 ^d		0.37 ± 0.10 ^c	
	Cotyledon	1.86 ± 0.39 ^{de}	41.3%	0.16 ± 0.04 ^d	15.7%	0.19 ± 0.05 ^c	18.9%
	Seed coat	26.40 ± 1.13 ^c	58.7%	8.61 ± 1.28 ^c	84.3%	8.16 ± 0.54 ^b	81.1%

¹N=3

²Means with different superscripts within each column are significantly different (P<0.05)

³TPC: expressed as mg/g gallic acid equivalents (GAE) of dry weight (DW) sample; CTC: expressed as mg/g cyanidin equivalents (CE) of dry weight sample; TFC: expressed as μ g/g catechin equivalents (CE) of dry weight samples

⁴Contributions of seed coat and cotyledon to TPC, CTC and TFC to the total content in seed were calculated as cotyledon accounts for 90% of the total seed weight and seed coat accounts for 10% of the total seed weight.

⁵Heat treatment here used infrared heating to the surface temperature of 150 °C.

The red lentil seed coat contained more ($P<0.05$) extractable total phenolics, condensed tannins and total flavonoids than that of the whole seed and cotyledon using either water or aqueous ethanol as the solvent (Table 3.4). Of the seed coat extracts of red lentil, the aqueous ethanolic extracts had total phenolic content (TPC) of 33.24 to 38.21 mg/g DW GAE, condensed tannin level (CTC) of 37.57 to 43.54 mg/g DW CE and total flavonoid contents (TFC) of 10.49 - 10.90 $\mu\text{g/g}$ DW CE. The concentration of phenolic compounds recovered from seed coat was higher ($P<0.001$) in ethanolic extracts than water extracts. The CTC in seed coat was around 12 to 622 times higher than cotyledon and whole seed and TFC in seed coat was around 15 to 77 times higher than cotyledon and whole seed from 70% (v/v) ethanol extracts. When the values are compared with water extracts (Table 3.4), cotyledons released more or less the same amount of phenolics to 70% (v/v) ethanol, which was also the case for whole seed. In small red lentil seed, the quantitative contribution of cotyledons to the total seed weight was 90% while the seed coat was 9 - 10%. So it can be expected that there is a very slight difference in TPC of cotyledon and the whole seed. In a study conducted by Xu, Yuan and Chang (2007), the TPC of whole seed of various lentil cultivars (planted in northern US) ranged from 4.86 to 9.60 mg/g gallic acid equivalents, the TFC ranged from 3.04 to 4.54 mg/g catechin equivalents and the CTC ranged from 3.73 to 10.20 mg/g catechin equivalents. Due to the differences in cultivars, and the composition of solvents (aqueous acetone vs aqueous ethanol) used in the present study, it is difficult to compare with the reported values by Xu et al. (2007). The same authors compared different solvent systems, such as 50% and 80% (v/v) acetone, acidified 70% (v/v) acetone, 70% (v/v) methanol, 70% (v/v) and ethanol and found that the organic solvent type and composition can have a significant effect on extracting phenolic compounds from lentil seeds. According to their study, the extraction efficiency of these solvent systems for phenolic components of red lentil can be listed as: acidified 70% (v/v) acetone > 80% (v/v) acetone > 50% (v/v) acetone > 70% (v/v) methanol > 70% (v/v) ethanol > anhydrous ethanol. In the present study, solvents such as acetone were not considered as the extraction medium because the later studies are related to

muscle protein myoglobin. It was observed that the heme molecule could easily dissociate from the myoglobin protein in the presence of acetone causing a faster oxidation (Wedzicha & Ladikos, 1986). In a recent study by Zhang et al. (2015), the results showed that 70% (v/v) acidified methanol as an extraction medium resulted in TPC of 6.60 mg/g gallic acid equivalents, TFC of 1.50 mg/g catechin equivalents and CTC of 5.46 mg/g catechin equivalents for red lentil (*var.* Maxim). The higher amount of condensed tannins reported in the study by Zhang et al. (2015) may be related to the differences in the external standard used (catechin vs cyanidin) in the present study. Cyanidin is believed to give higher absorbance values when acidified butanol is used in the assay (Schofield, Mbugua, & Pell, 2001). Overall, the contribution of TPC and CTC from cotyledon and seed coat vary depending on which solvent is used and on whether heat treatment is applied.

The TPC, CTC of 70% (v/v) ethanol extracts of seed coat from raw lentil showed slight but statistically higher ($P < 0.05$) levels than those from heat-treated lentil (Table 3.4). However, the extracts of cotyledon and whole seeds did not show a change for these compounds due to heat treatment in either water or ethanolic extracts. Reduction in total phenolic content of red lentil seeds (25% for Pardina cultivar but no change for Crimson cultivar) due to cooking at 98 °C for 30 min was reported by Han and Baik (2008). However, soaking the seeds at 22 °C for 12 h in water brought a 15 - 30% reduction in the TPC of both cultivars suggesting a material loss due to removal of soaked water. Aguilera et al. (2010) also found that soaking and boiling for 30 min can lead to a decrease in some phenolic compounds, such as catechins, procyanidins, flavonols and flavones. It is not surprising that some of the water soluble compounds could dissolve in water and be lost from lentil seeds during these processes. Compared with soaking and boiling, dry heat processing such as using infrared heat can reduce material loss and provide heat treatment in a fast and continuous way.

The biological functions of phenolic compounds in seed include: increasing hardness of the seed, maintaining hydration and preventing microbial growth so they are mainly located in seed

coat as a protector of the seed (Yasseen, Barringer, Splittstoesser, & Costanza, 1994). In the study conducted by Oomah et al. (2011), the ethanol extractable TPC of the hull of Canadian grown red lentil was approx. five times higher than whole seed, and water extractable phenolics were three times higher. A study conducted in later years with different Canadian cultivars (Boudjou, Oomah, Zaidi, & Hosseinian, 2013) observed that TPC of ethanolic extracts of red lentil hull is over nine times higher than similar extracts of whole seed. This study corroborates with the literature and shows that lentil seed coat contains phenolic compounds soluble in water or aqueous ethanolic solutions.

From our observation, seed cotyledon and seed coat account for 90% and 9% of the seed weight, respectively therefore the contribution of each seed physical component to TPC, CTC and TFC is different (Table 3.4). Considering the phenolic compound based antioxidant capacity, red lentil seed coat accounts for much higher potential than cotyledons or the whole ground seed. The aqueous ethanolic extracts brought more seed coat phenolics to solution than from cotyledons. When water was the solvent, more phenolics of red lentil cotyledons were extracted providing potentially better antioxidant capacity than its ethanolic extract. When weight proportion of cotyledon and seed coat were considered for water extracts, cotyledon contributes 62.3% TPC to raw lentil seeds and 41.3% TPC for heat-treated lentil seeds (Table 3.4). These numbers were much higher than their contribution to the TPC of ethanolic extracts (about 10 to 13%) and were comparable to seed coat's contribution to the TPC of water extracts.

The presence of water soluble phenolic compounds brings another positive feature for the lentil flour as a binder in meat, indicating possible antioxidative effect on raw meat products. In the previous studies of our group, incorporation of heat-treated lentil flour in raw beef burgers can prolong the redness, slow down lipid oxidation in refrigerated conditions (Der, 2010; Pathiratne, 2014). Such potent antioxidant effect of lentil flour could be attributed to the high amount of water soluble phenolic compounds and the deactivation of oxidative enzymes by heat treatment.

3.4.6 Myoglobin and unsaturated lipid containing assays

Understanding the antioxidant ability of the phenolic/protein extracts on oxidation of myoglobin and unsaturated lipids is highly relevant to describe the possible interference of the constituent components on the colour changes and lipid oxidation occurring in a fresh meat system. In these assays, both ethanolic extracts and water extracts were evaluated using model systems.

3.4.6.1 Metmyoglobin reducing assay

The changes of metmyoglobin level due to each test component over an 8 h period are shown in Table 3.5 and Table 3.6. Note that lower metmyoglobin level indicates higher reduction ability of lentil extracts. The results showed that the extraction solvent has a significant effect ($P < 0.05$) on the compounds that provide the reducing ability of metmyoglobin. As shown in Table 3.6, the 70% ethanol extract lowered the metmyoglobin level for all samples, compared with the samples using water as solvent (Table 3.5). It is believed that ethanol can reduce the metmyoglobin and hypervalent ferrylmyoglobin to oxymyoglobin in the model system (Harada, Tamura, & Yamazaki, 1986). When ethanolic extracts were considered, the lowest values for metmyoglobin level (highest reduction occurred) for lentil seed coat samples were at 1 h and 2 h, and beyond that the values remained unchanged. The levels of metmyoglobin remained much lower ($P < 0.01$) for the assay mixtures containing ethanolic extracts of seed coat (52.8 - 63.9%) than cotyledons (69.7 - 84.0%) (Table 3.6). There was only a slight difference between the components extracted from heat-treated vs. raw, also the increase in concentration has only a slight effect (Table 3.6). It was observed that the standard deviations of the ethanolic extracts are higher than water extracts, probably because the volatility of ethanol, that may have caused variability of the determination.

Table 3.5 Changes of metmyoglobin level in the metmyoglobin reducing model with each lentil extracts (water) over 8 h period at room temperature (Mean \pm SD)

Treatment ¹				Level of water extractable TPC ($\times 10^{-2}$ mg/mL assay)	Time				
					30s	1h	2h	4h	8h
Control-water					88.3 \pm 1.1	91.3 \pm 0.9	92.7 \pm 0.7	92.9 \pm 0.5	94.1 \pm 1.5
Ascorbic acid ²					75.7 \pm 14.8*	58.2 \pm 9.9*	56.9 \pm 9.5*	59.0 \pm 4.5*	63.2 \pm 1.8*
LOX ²					93.2 \pm 0.3	94.4 \pm 0.3	95.0 \pm 0.3	95.2 \pm 0.3	96.4 \pm 0.5
SOD ²					95.1 \pm 0.8	97.3 \pm 0.3	98.2 \pm 0.4	98.5 \pm 0.5	99.0 \pm 0.3
Cotyledon	Raw	94 μ L	1.28		92.7 \pm 1.6	94.7 \pm 0.5	95.5 \pm 1.2	95.5 \pm 1.2	94.8 \pm 2.5
		188 μ L	2.56		90.7 \pm 1.9	92.9 \pm 2.0	94.0 \pm 2.2	94.2 \pm 1.4	92.9 \pm 3.0
		375 μ L	5.10		87.9 \pm 2.5	90.4 \pm 4.8	91.2 \pm 4.7	91.3 \pm 4.3	90.2 \pm 4.9
	Heat-treated	94 μ L	0.53		91.8 \pm 0.1	93.6 \pm 0.4	94.8 \pm 0.2	95.1 \pm 0.7	96.4 \pm 0.7
		188 μ L	1.05		92.3 \pm 0.4	93.8 \pm 0.3	95.2 \pm 0.0	95.9 \pm 0.6	96.7 \pm 0.4
		375 μ L	2.10		92.2 \pm 0.5	93.5 \pm 0.4	94.6 \pm 0.3	95.8 \pm 0.4	96.3 \pm 0.5
	Seed coat ³	Raw	94 μ L	7.14	92.8 \pm 1.0	73.9 \pm 1.9*	71.3 \pm 1.7*	74.8 \pm 2.3*	86.8 \pm 4.5
			188 μ L	14.29	93.1 \pm 0.6	72.4 \pm 3.9*	70.9 \pm 3.0*	76.1 \pm 2.1*	87.1 \pm 2.6
			375 μ L	28.50	91.8 \pm 0.4	76.5 \pm 4.2*	75.4 \pm 3.6*	79.5 \pm 2.9*	85.1 \pm 2.3
		Heat-treated	94 μ L	7.52	89.8 \pm 3.5	70.7 \pm 1.6*	69.6 \pm 1.9*	74.1 \pm 2.4*	85.0 \pm 5.7
			188 μ L	15.04	89.1 \pm 4.7	66.6 \pm 3.6*	68.3 \pm 2.7*	75.7 \pm 2.4*	84.9 \pm 3.4
			375 μ L	30.00	87.9 \pm 4.8	69.3 \pm 4.0*	72.4 \pm 2.3*	79.3 \pm 2.7*	84.1 \pm 2.3

¹RT: room temperature, N=3

²Lipoxygenase (LOX): 4.78×10^6 units/mL of the assay; superoxide dismutase (SOD): 4.66×10^3 units/mL of the assay; ascorbic acid: 0.5 mg/mL of the assay.

³* indicates significant difference (P<0.05) of metmyoglobin level from Control-water within the column.

Table 3.6 Changes of metmyoglobin level in the metmyoglobin reducing model with each lentil extracts (70% ethanol) over 8 h period at room temperature (Mean \pm SD)

Treatment ¹		Level of addition	Level of 70% ethanol extractable TPC ($\times 10^{-2}$ mg/mL assay)	Time				
				30s	1h	2h	4h	8h
Control-ethanol				82.2 \pm 2.2	84.0 \pm 1.1	83.4 \pm 1.8	82.1 \pm 1.7	80.6 \pm 1.0
Control-water				88.3 \pm 1.1	91.3 \pm 0.9	92.7 \pm 0.7	92.9 \pm 0.5	94.1 \pm 1.5
Catechin ²				83.8 \pm 1.3	81.5 \pm 1.3	78.7 \pm 1.0	75.2 \pm 0.8	72.8 \pm 0.3
Cotyledon	Raw	94 μ L	0.12	79.2 \pm 2.3	77.8 \pm 2.4	76.2 \pm 3.0	74.7 \pm 3.6	73.6 \pm 4.2
		188 μ L	0.24	80.4 \pm 9.6	80.1 \pm 10.4	79.7 \pm 11.6	79.5 \pm 12.7	79.1 \pm 12.9
		375 μ L	0.49	76.8 \pm 9.7	76.5 \pm 11.5	76.0 \pm 12.1	75.6 \pm 12.8	74.7 \pm 12.8
	Heat-treated	94 μ L	0.15	83.7 \pm 6.1	84.2 \pm 7.4	84.0 \pm 8.9	83.6 \pm 9.9	83.5 \pm 10.7
		188 μ L	0.30	81.5 \pm 8.6	81.8 \pm 9.7	81.8 \pm 11.1	81.5 \pm 12.2	81.0 \pm 12.9
		375 μ L	0.59	73.8 \pm 2.7	70.5 \pm 1.9	69.7 \pm 2.0	69.0 \pm 2.1	67.6 \pm 2.3
	Seed coat ³	Raw	94 μ L	72.1 \pm 2.0	64.2 \pm 3.3	63.9 \pm 4.1	64.4 \pm 4.8	65.1 \pm 5.7
			188 μ L	69.9 \pm 1.1	63.1 \pm 1.9	62.6 \pm 2.2	62.2 \pm 2.7	61.0 \pm 3.2
			375 μ L	67.9 \pm 9.1	58.3 \pm 8.5*	58.6 \pm 10.0	58.1 \pm 11.2	57.0 \pm 10.9
		Heat-treated	94 μ L	71.4 \pm 3.8	63.4 \pm 4.9	63.1 \pm 5.1	63.5 \pm 5.5	64.4 \pm 6.1
			188 μ L	69.9 \pm 2.5	62.6 \pm 5.3	61.7 \pm 5.4	62.4 \pm 5.9	61.9 \pm 6.2
			375 μ L	63.3 \pm 6.2	53.7 \pm 3.3*	52.8 \pm 3.0*	52.2 \pm 2.8*	51.1 \pm 1.3*

¹RT: room temperature, N=3

²Catechin: 0.21 mg/mL of the assay.

³* indicates significant difference (P<0.05) of metmyoglobin level from Control-ethanol within the column.

When the water extracts of the same materials are considered, they could not lower the metmyoglobin content assay mixture to the level that the ethanolic extracts of the same material did (around 60% metmyoglobin). But water extracts of seed coat were able to pose strong reducing ability that can lower the metmyoglobin content below 70% in 2 h after the assay started but lost the power later (shown in Table 3.6 and Figure 3.3). Also, the heat treatment or increase in concentration was not effective in reducing metmyoglobin content of the assay. This might suggest that the reducing ability of phenolic compounds on metmyoglobin is also affected by specific phenolics present. When both these extracts are compared for sustaining the metmyoglobin reducing ability over the 8h period, water extracts showed an increase in metmyoglobin percentage% after the lowest value at 1 or 2 h, while ethanolic extracts maintained the low values (Table 3.5). The comparatively greater metmyoglobin reducing ability of ethanolic extracts than water extracts may be due to the effect of dissolved compounds and the solvents. The ethanolic extracts of lentil contained higher TPC and CTC than the water extracts (Table 3.4). The higher content of these phenolic compounds may contribute to stronger metmyoglobin reducing capability than the ones with lower content.

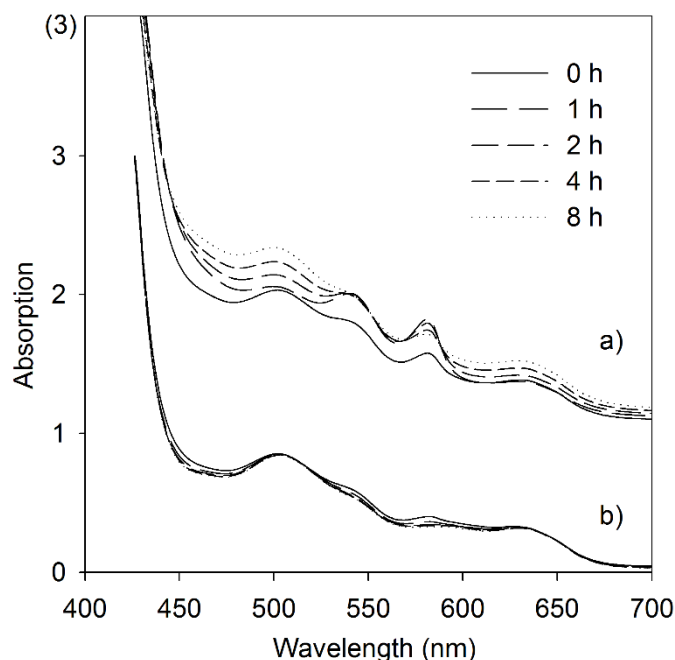


Figure 3.3 The changes in absorption spectrum over 8 h period. The peaks at 582 nm and 538 nm are the characteristic absorptions of oxymyoglobin, while the peak at 500 nm is the characteristic absorption of metmyoglobin. a) addition of water soluble, heat-treated seed coat extract; 188 μ L, b) control-water

Other researchers also found that the reducing ability of phenolic compounds on metmyoglobin can depend on concentration and molecular species. According to Miura et al. (2014), 2'-S-cysteinylcaffeic acid (a phenolic compound, 15 to 600 μ M) caused an increase in the oxymyoglobin production from metmyoglobin in a concentration dependant manner. In their research, a higher level of phenolic compounds caused higher conversion of metmyoglobin. In a separate study, Inai et al. (2014) observed that pure phenolic compounds, such as catechin, kaempferol and resveratrol caused different extents of metmyoglobin reduction. For example, catechin at 600 μ mol/L added to 120 μ mol/L metmyoglobin can form 20% oxymyoglobin (after incubation at 37 $^{\circ}$ C for 2 h) while kaempferol and quercetin at 300 μ mol/L can achieve higher oxymyoglobin level than catechin at 600 μ mol/L. It was found that phenolic compounds can directly reduce metmyoglobin to form oxymyoglobin, and the phenolic compounds are converted

to quinones (Inai et al., 2014). In addition, according to the results of Dueñas et al. (2006), the difference in the phenolic compounds found in lentil seed coat and cotyledon is not only their levels, but the types as well. Seed coat contains more catechins, dimer procyanidins, trimer procyanidins and prodelphinidins while cotyledon contains more hydroxycinnamic acids (Dueñas et al., 2006). This difference in the type of molecules present may have contributed to the higher ability of seed coat extracts to reduce more metmyoglobin than the cotyledon extracts. All of these results suggest the seed coat may be the major contributor to prolong oxymyoglobin status and provide colour protection effect of lentil when mixed with raw minced meat.

There was no difference ($P>0.05$) found between the extracts of raw and heat-treated lentil seed on the metmyoglobin reducing capability (Table 3.6). Previous sections of this study showed the effect of heating treatment is mainly to reduce the enzyme activity, such as that of lipoxygenase and peroxidase, rather than affecting the extractability or content of phenolic compounds. According to the review by Nayak et al. (2015), cooking has mixed effects on the phenolic antioxidants because a decrease was observed in the original phenolic compounds and an increase in new phenolic compounds were observed during cooking of grain foods. This could lead to the similar metmyoglobin reducing ability between heat-treated and raw lentil extracts. Also, different addition levels of seed coat water extracts did not show significant difference ($P>0.05$) on metmyoglobin reducing ability, suggesting a maximum value for the metmyoglobin reducing ability is present.

Addition of either lipoxygenase and superoxide dismutase did not have an effect on the reduction of metmyoglobin. Therefore, it is reasonable to anticipate that myoglobin is not their direct substrate. Lipoxygenase can catalyze the hydroperoxidation with one molecular oxygen of polyunsaturated fatty acids such as linoleic acid while superoxide dismutase can scavenge the reactive oxygen species (Khalyfa et al., 1990). These enzymes have indirect effects on lipid oxidation and oxidation of myoglobin. The assay used here involved only metmyoglobin without the addition of lipids. Catechin added at 0.21 mg/mL level was found to reduce metmyoglobin to

73%. Ascorbic acid, a potent antioxidant, also has a stronger metmyoglobin reducing ability and lowered the metmyoglobin level to as low as 56% when tested at 0.5 mg/mL of the assay. This is a very strong antioxidant effect on metmyoglobin reducing ability and possibly on meat colour protection as well. Overall, the results suggest that lentil seed coat can protect meat colour by reducing metmyoglobin to oxymyoglobin, while cotyledon does not show such ability.

3.4.6.2 Metmyoglobin induced linoleic acid oxidation assay

The catalytic effect of metmyoglobin on unsaturated fatty acid oxidation and the inhibitory effect by lentil extracts can be observed via the linoleic acid oxidation assay. The assay mixture used for this test contained both metmyoglobin and free linoleic acid. The extracts (both water and ethanolic) of lentil seed coat and cotyledon slowed down the rate of linoleic acid oxidation induced by metmyoglobin at different levels (Table 3.7). Metmyoglobin acts as an initiator of linoleic acid oxidation, producing conjugated dienes which can be monitored by absorbance at 234 nm. Kühn et al. (1981) discovered that the addition of met-hemoglobin dramatically increased the oxidation rate of linoleic acid oxidation, revealing a quasi lipoxygenase activity of metmyoglobin. Rao, Wilks, Hamberg and Ortiz de Montellano (1994) further established that the heme crevice of the myoglobin molecule is the active site for the oxidation of linoleic acid. The reaction might be occurring with the iron species such as ferryl oxygen within the heme molecule. Observation of the present study for the control assay mixtures without (1 unit) and with (1002.8 unit) metmyoglobin shows the accelerating effect of metmyoglobin in linoleic acid oxidation (Table 3.7). This also suggests an interaction between oxymyoglobin oxidation and lipid oxidation, that is, the products of oxymyoglobin oxidation can induce/initiate the oxidation of unsaturated fatty acid.

Table 3.7 Oxidation rate of linoleic acid initiated by metmyoglobin with aqueous and ethanolic extracts of red lentil seed components with or without heat treatment (Mean \pm SD)

Treatment ^{1,2}		Addition level (μL)	Level of 70% ethanol extractable TPC (×10 ⁻⁴ mg/mL assay)	Level of water extractable TPC (×10 ⁻⁴ mg/mL assay)	Activity (Change of 0.001 A ₂₃₄ per minute)	
Control (Without MetMb)					1.0 ± 1.2 ^f	
Control (With MetMb)					1002.8 ± 89.1 ^a	
Ascorbic acid (25 mg/mL)		5			0.0 ± 0.0	
		10			0.0 ± 0.0	
Catechin (0.54 mg/mL)		10			638.9 ± 24.8	
		25			570.2 ± 12.8	
		50			470.9 ± 23.6	
<i>Red lentil</i>					70% (v/v) ethanol	Water
Cotyledon extracts	Raw	10	0.35	3.70	952.2 ± 34.2 ^{ab}	769.2 ± 60.0 ^{bc}
		25	0.88	9.24	1003.3 ± 64.3 ^a	796.7 ± 80.7 ^{ab}
		50	1.76	18.48	1025.2 ± 50.4 ^a	766.0 ± 136.9 ^{bc}
	Heat-treated	10	0.43	1.52	951.7 ± 84.5 ^{ab}	805.6 ± 166.3 ^{ab}
		25	1.07	3.80	948.8 ± 94.5 ^{ab}	837.3 ± 123.4 ^{ab}
		50	2.13	7.61	967.3 ± 89.7 ^{ab}	766.1 ± 104.4 ^{bc}
Seed coat extracts	Raw	10	30.57	20.65	308.9 ± 59.7 ^{de}	540.4 ± 82.2 ^{cd}
		25	76.43	51.63	0.0 ± 0.0 ^f	270.7 ± 44.7 ^e
		50	152.86	103.26	0.0 ± 0.0 ^f	132.0 ± 15.3 ^{ef}
	Heat-treated	10	27.26	21.74	266.3 ± 43.3 ^e	523.7 ± 12.9 ^d
		25	68.16	54.34	137.0 ± 6.1 ^{ef}	266.6 ± 34.1 ^e
		50	136.32	108.70	0.0 ± 0.0 ^f	87.1 ± 44.7 ^{ef}

¹N=3

²Means with different superscripts within each column are significantly different (P<0.05)

Seed coat extracts from either water or 70% ethanol (v/v) slowed down ($P < 0.05$) the oxidation rate of linoleic acid compared with the control with metmyoglobin (Table 3.7). And extracts of 70% ethanol (v/v) showed stronger ($P < 0.05$) inhibition effect than those of water extracts. Heat treatment basically had no effect on the extracts of seed coat in inhibition of linoleic acid oxidation. Cotyledon extracts of 70% ethanol (v/v) cannot inhibit ($P > 0.05$) the oxidation of linoleic acid, but the water extracts of cotyledon added at 50 μL exhibited some inhibition effect compared with the control. Heat treatment also did not show any effect on cotyledon extracts to inhibit linoleic acid oxidation initiated by metmyoglobin. The inhibition ability of seed coat should be attributed to the high level of phenolic compounds. Similar ability of phenolic compounds to slow down linoleic acid oxidation induced by metmyoglobin has been reported previously. Goupy et al. (2009) showed that red wine pigment, mainly anthocyanins can decrease linoleic acid oxidation when metmyoglobin was the oxidation initiator. Also, a concentration based difference can be observed for seed coat samples since higher levels of addition of seed coat extracts can further slow down the oxidation of linoleic acid than lower levels. A similar concentration based difference was observed with the treatment of catechin, a common phenolic compound found in lentil and green tea in a similar assay model (Graham, 1992; Zhang et al., 2015).

One of the proposed mechanisms for how phenolic compounds can slow down the oxidation of linoleic acid initiated by metmyoglobin is that phenolic compounds reduce the metmyoglobin (Fe^{3+}) to oxymyoglobin (Fe^{2+}) that results in losing the ability to initiate lipid oxidation as shown in section 3.4.6.1. Another possible reason is that phenolic compounds could stop the propagation of lipid oxidation by scavenging lipid peroxy radicals. Moreover, phenolic compounds have been found to inhibit the activity of lipoxygenase. Banerjee (2006) found that phenolic compounds from green tea as well as other antioxidants can inhibit the lipoxygenase activity. In that study, different types of phenolic compounds were added to the mixture of lipoxygenase extracted from mackerel and linoleic acid as the substrate. Epigallocatechin gallate (EGCG), epicatechin gallate

(ECG), epicatechin (EC), epigallocatechin (EGC) and catechin (sorted in descending order of inhibition capability) were able to inhibit the oxidation of linoleic acid initiated by lipoxygenase. It was suggested that flavonoids (a type of phenolic compound) could be oxidized to quinone during the formation of lipid peroxy radical catalyzed by lipoxygenase (Sadik, Sies, & Schewe, 2003). The formed quinone can then bind to sulfhydryl or amino groups on the enzyme leading to an inhibitory effect on catalytic activity of the enzyme (Sadik et al., 2003). Since myoglobin is a protein in meat, phenolic compounds may also perform similarly to inhibit its catalytic effect on linoleic acid oxidation. The results suggests that phenolic compounds from lentil can inhibit the oxidation of linoleic acid initiated by metmyoglobin, indicating a possible way of lentil flour to retard lipid oxidation in raw meat products.

3.5 Summary and Conclusions

Lentil cotyledons contained a higher protein content than the seed coat while fat and ash contents in both parts are not different. The distribution of enzyme and antioxidant activities of soluble proteins recovered from cotyledon and seed coat were different for lipoxygenase, peroxidase and glutathione reductase being mainly located in the cotyledon, but higher superoxide dismutase activity was found in the seed coat. Both cotyledon and seed coat contributed to the antioxidant activity of the lentil seed either by providing antioxidative enzymes or the phenolic compounds and protein-bound phenolics. In the model systems, antioxidative compounds was able to reduce metmyoglobin to form oxymyoglobin when lipids were not present. Since metmyoglobin can increase the rate of unsaturated lipid oxidation in a complex system such as meat, the ability to reduce metmyoglobin could be an advantage. Lentil seed coat extracts were successfully able to lower the oxidation rate of linoleic acid in the presence of metmyoglobin. Heat treatment using infrared heating to 150 °C was able to deactivate oxidative enzymes (LOX and POX) without affecting superoxide dismutase activity. Heat treatments also caused an increase in the metal chelating ability of the soluble proteins of lentil. These results suggested that heat treatment has an overall beneficial effect on the lentil seed to retard lipid

oxidation either in lentil seed itself or potentially in the food system that incorporates lentil. Infrared heat treatment could be a preferred method of precooking treatment as it has a shorter cooking time for lentil seeds that can improve vital functional antioxidant attributes when used as a binder in fresh meat products.

3.6 Connection to the next chapter

From the results shown above, it is evident that oxidative enzymes are mainly located in the seed cotyledon of lentil and they are greatly affected by heat treatments above 90 °C. The oxidative enzymes are able to accelerate lipid oxidation by catalyzing the production of hydroperoxides (St. Angelo, Kuck, & Ory, 1979). Since lipid oxidation and myoglobin oxidation are interactive (Faustman et al., 2010), it can be assumed that with higher rates of lipid oxidation by adding active exogenous oxidative enzyme to meat products, lipid oxidation can be promoted, as well as myoglobin oxidation, as a result, causing meat discoloration.

On the other hand, the lentil seed coat provides more antioxidant activity than the cotyledon (on a w/w basis) according to our study, coming from heat-resistant antioxidant enzymes such as superoxide dismutase (SOD). The results of antioxidant assays indicate existence of higher antioxidant activity in lentil seed coat rather than in seed cotyledon which is related to the high total phenolic content of seed coat. Since the seed coat extracts can cause reduction of metmyoglobin and also prevent linoleic acid oxidation initiated by metmyoglobin, it is reasonable to speculate that the seed coat part of lentil could play a major role in meat colour protection and slowing down lipid oxidation. However, no detailed information is available on the impact of different lentil seed components on meat colour and lipid oxidation in a real meat system.

Therefore, an applicable way to examine the contributions of different physical components of lentil seed to meat colour protection and lipid oxidation prevention would be using a meat product model, especially with comminuted meat particles such as beef burgers in which both these key reactions occur concomitantly.

4 STUDY 2 APPLICATION OF THE ANTIOXIDANT ABILITY OF LENTIL FLOUR TO PROTECT MEAT COLOUR AND UNSATURATED LIPIDS DURING REFRIGERATED STORAGE OF FRESH BEEF BURGERS

4.1 Abstract

The oxidative stability of meat pigments and unsaturated lipids of raw ground beef was investigated by incorporating flours of raw and heat-treated lentils and their physical components, namely whole seed (at level of 6.0%), cotyledon (at 5.4%) and seed coat (at 0.6%) as binders in beef burger formulations. Surface colour, surface myoglobin states and the lipid oxidation determined as thiobarbituric acid reactive substances (TBARS) of beef burgers were monitored during 7 days of refrigerated storage at 4 °C. Results showed that the addition of heat-treated lentil flour to fresh ground beef resulted in higher a^* value ($P<0.05$) and lower metmyoglobin ($P<0.05$) than raw lentil flour, but no difference was found for TBARS. The TBARS values of burgers with either raw or heat-treated lentil flour (whole seed) showed lower ($P<0.05$) values than the control. Seed coat flour separated from raw lentil seed added to beef burgers caused higher a^* values ($P<0.05$), lower metmyoglobin ($P<0.05$) and lower TBARS ($P<0.05$) than burgers with added cotyledon flour or the control formulation. Cotyledon flour from raw lentil, on the contrary, caused a faster discoloration of the meat and comparable development of TBARS level as the control. However, the seed coat and cotyledon separated from heat-treated lentil seeds protected a^* value and lowered TBARS of beef burgers, offering strong antioxidant activity. Negative correlations were found between redness and metmyoglobin and between redness and TBARS values. The results suggested that heat-treated lentil flour provided antioxidant effect capable of protecting colour and preventing unsaturated lipid oxidation.

4.2 Introduction

Colour is an important property of fresh meat products. To consumers, the colour is an indicator of wholesomeness in fresh meat (Suman & Joseph, 2013) and has strong correlation with their decision to purchase that product (Carpenter et al., 2001). When colour of meat changes, myoglobin as the major pigment tends to oxidize from its oxy- form (bright red colour) to met- form (brown colour) (Mancini & Hunt, 2005). Myoglobin oxidation can also induce oxidation of unsaturated lipids and as a result, lead to off-flavour development of meat products (Ramanathan et al., 2009). The products of lipid oxidation such as 4-hydroxynonenal (4-HNE) could in turn accelerate the oxidation of oxymyoglobin (Lynch & Faustman, 2000), therefore making these reactions inter-related and inter-dependant.

In order to improve meat colour stability and inhibit lipid oxidation, many researchers have incorporated antioxidants of either synthetic or natural origin into raw meat products. Sodium erythorbate, as the salt of ascorbic acid isomer, has proven as highly effective in redness protection of raw beef patties compared at a 0.04% (w/w) level with the control formulation without any additive under 4 °C storage for 48 h (Suman et al., 2005). In fact, sodium erythorbate, erythorbic acid, sodium ascorbate and ascorbic acid, when added into ground beef at 2.3 mmol/L, all were able to protect ground beef colour compared with the control after 48 h storage at 4 °C (Sepe et al., 2005). Among the antioxidants of natural sources, Yu et al. (2010) added 0.02% to 0.1% peanut skin extract into ground beef and found that 0.1% addition of extract slowed down the decrease of a^* value during a 12-day refrigerated storage. Vargas-Sánchez et al. (2014) added propolis (an exudate collected by honey bees from tree buds) extracts at 2% into beef patties and stored those patties at 2 °C for 8 days. It was found that the addition of three types of propolis extract could successfully slow down discoloration of beef patties, and the extract with higher amount of phenolic compounds caused higher a^* value of beef patties on day 8 of storage. Such applications of natural extracts to protect colour of raw meat products take advantage of the rich amount of antioxidants, mainly phenolic compounds in these extracts.

Previous studies conducted in our group found that adding 6.0% w/w heat-treated lentil flour as a binder can protect colour of raw beef burger in refrigerated storage condition while the raw lentil flour not only cannot, but accelerated discoloration of beef burgers (Der, 2010; Pathratne, 2014).

Lentil is an important legume crop with a high amount of protein, starch and dietary fibre, but low fat content (Candela et al., 1997; Faris et al., 2013). This feature makes lentil flour a potential binder for meat products. Previous studies in our lab have shown that incorporation of lentil flour into beef burgers at 6.0% w/w can significantly improve cook yield and moisture retention (Der, 2010). These functional characteristics are comparable to that observed from traditional binders used in Canada, such as toasted wheat crumb (6.0% w/w) which is an extruded crumbled wheat dough made with wheat flour and water (Der, 2010). Raw lentil seed can be heat treated prior to any further application in order to shorten cooking time and to increase the digestibility and availability of nutrients (Satya et al., 2010). Infrared heating is a type of heat treatment that uses infrared radiation from wavelengths of 1.8 - 3.4 μm to dry heat materials that are exposed to it (Zheng et al., 1998). Heat-treated lentil contains lower levels of volatile compounds that are related to the beany aroma of lentil flour, which is a benefit when used as an ingredient in meat products (Shariati-Ievvari et al., 2016). More importantly, applying infrared heating can greatly decrease the activity of oxidative enzymes, such as lipoxygenase and peroxidase which could accelerate lipid oxidation either in lentil seed itself or in meat products (Pathiratne et al., 2015). But heat treatment does not affect the activity of superoxide dismutase in lentil which catalyzes the dismutation of the superoxide radicals and provides antioxidant functionality in lentil and potentially in food systems (see section 3.4.2.1).

As an added benefit, lentil contains considerable amount of phenolic compounds, such as flavonoids, lignins and tannins (Amarowicz & Pegg, 2008) and the total phenolic content in lentil is higher than other food legumes, such as pea, chickpea, soybean, kidney and black bean (Oomah et al., 2011). Because of the high content and variety of phenolic compounds present, Xu and Chang (2007) and Han and Baik (2008) believed that lentil should exhibit high antioxidant

activity. That suggests that incorporation of heat-treated lentil flour may help in inhibiting colour change and lipid oxidation of the fresh ground meat products. As an old practice, red lentil is always dehulled before home consumption to improve palatability, appeal and cooking performance (Wang, Hatcher, Toews, & Gawalko, 2009). Lentil seed can be physically separated into the seed coat (~9% weight) and cotyledon (~90% weight) (Dueñas et al., 2002). The lentil seed coat contains a higher level of phenolic compounds than the cotyledon while cotyledon contains higher protein content (Dueñas et al., 2002). However, little is known about which physical component of lentil (with or without heat treatment) contributes more to colour protection and inhibition of lipid oxidation when incorporated into the raw meat products.

Therefore, this study was designed to evaluate the effect of physical components with and without heat treatment (infrared heating) of lentil seeds on the colour, myoglobin redox states and lipid oxidation of ground beef in burger formulations that were stored under refrigeration conditions. The cotyledon and seed coat flour were added as their natural proportion (90% and 9%, respectively) of the whole seed, trying to figure out which part contributes more antioxidant activity.

4.3 Materials and Methods

4.3.1 Raw materials and chemicals

Small red lentil (CDC Maxim) that was harvested in 2013 and received in May, 2014 was used in the study. Incoming seeds were stored in bags at room temperature prior to use. Vacuum packaged fresh beef tops and bottom rounds (Canadian AA or AAA Grade) were obtained from Cargill (High River, AB). For all the three replicated product preparations, meat was received (on different days for each replication) and used within 21 to 45 days of animal slaughter. Incoming meat was stored at 1 °C before use and only beef with normal pH (~5.6) was used. Sodium erythorbate, salt (containing >99.2% sodium chloride, Windsor[®] Salt, The Canadian Salt Company Ltd.), toasted wheat crumb (11.6 g protein, 1.2 g fat and 77.0 g carbohydrate per 100 g,

Biscrumb coarse, Griffith Laboratories) used in the formulation were all food grade.

Trichloroacetic acid (TCA), thiobarbituric acid (TBA) and 1, 1, 3, 3,-Tetramethoxypropane employed in chemical analyses were of ACS grade.

4.3.2 Heat treatment of lentil

Prior to heat treatments, lentil seeds were tempered as described in section 3.3.2.1. The tempered seeds were then heat treated using infrared heat treatment conducted at InfraReady Products (1998) Ltd. (Saskatoon, SK Canada) as described in section 3.3.2.2. The seeds were heat treated to a surface temperature of 150 °C to completely deactivate oxidative enzymes. Heat-treated seeds and raw seeds were dried under ambient temperature for 12 h on trays after heat treatment (to moisture content of approximately 12%) and separated and ground as described in section 3.3.2.4.

4.3.3 Beef burger formulation and processing

Beef burgers were prepared with added binders, salt and ice water as shown in Table 4.1. Since the binder levels ranged from 0.6% to 5.4% and 6.0%, two corresponding controls with differing meat contents (control for low binder level and control for high binder level) were included.

Table 4.1 Treatment regime of lentil flours and formulations of beef burger

Treatments and binder levels	Meat g	Binder /Antioxidants g	Ice water g	Salt g	Total g
Control 1-low binder equivalent	85.0	0.0	14.1	0.9	100.0
Control 2-high binder equivalent	91.0	0.0	8.1	0.9	100.0
Toasted wheat crumb (TWC), 6.0%	85.0	6.0	8.1	0.9	100.0
Sodium erythorbate, 0.05%	85.0	0.05	14.05	0.9	100.0
Raw lentil, whole seed, 6.0%	85.0	6.0	8.1	0.9	100.0
Raw lentil, cotyledon, 5.4%	85.0	5.4	8.7	0.9	100.0
Raw lentil, seed coat, 0.6%	85.0	0.6	13.5	0.9	100.0
Heat-treated lentil, whole seed, 6.0%	85.0	6.0	8.1	0.9	100.0
Heat-treated lentil, cotyledon, 5.4%	85.0	5.4	8.7	0.9	100.0
Heat-treated lentil, seed coat, 0.6%	85.0	0.6	13.5	0.9	100.0

On the processing day, incoming meat was separated into meat blocks of lean and fat. Any visible connective tissues was removed and discarded. The lean and fat meat blocks were then ground through a 3/8 inch (9.5 mm) grinder plate (The Biro Manufacturing Co., Inc., Marblehead, OH). Randomly selected meat samples were obtained from the fat and lean blocks and were analyzed for fat content by an HFT 2000 rapid fat analyzer (Data Support Co., Encino, CA). The amounts of each meat block to obtain a final raw burger fat content of 17% was determined using a Pearson square calculation. The needed amounts of lean and fat meat to achieve 17% fat level were then mixed and ground through a 1/8 inch (3.2 mm) grinder plate. The ground meat, dry ingredients and icy water (0 °C) were transferred to a mixer bowl equipped with a flat paddle (Berkel BA-20 Mixer, Berkel Co., Countryside, IL) and mixed for 30 s at speed setting of one. Burger mixtures were then formed into 10-cm diameter patties (100 g) using a Hollymatic Patty Machine (Hollymatic Corp., Countryside, IL) stocked with patty paper. All processes were conducted below 4 °C in the University of Saskatchewan meat processing pilot plant. Meat temperature throughout the processing was monitored and maintained not to be

higher than 5 °C. The treatment order for processing was completely randomized, and all the equipment were cleaned thoroughly between batches. Burger production was conducted in triplicate from new sources of meat, with runs occurring eight days apart. Representative samples from each treatment were obtained and stored at -20 °C for compositional analysis. Four burgers of each treatment were placed individually on Styrofoam trays and overwrapped with oxygen permeable film (with moisture vapour transmission rate of 4 g/100 cm²/24 h at 20 °C and oxygen transmission rate of 81 mL/100 cm² at 20 °C, Vitawrap, Huntsman Packaging Co.). Burger samples were placed in an open chest retail display case at 4 °C under white light condition (~1300 lux) for 7 days. The positions of burger samples were rotated and changed every day during the storage period. Instrumental colour and chemical analysis were conducted on day 0, 1, 3, 5 and 7.

4.3.4 Compositional analyses and pH determination

Meat samples for compositional analyses were collected on each processing day and stored at -20 °C prior to use. For all chemical analyses, thawed samples were ground for 45 s using a food processor (Braun, Procter & Gamble, Toronto, ON) before use. Moisture, protein, fat and ash content were measured according to the methods of AOAC 950.46, 981.10, 960.39 and 920.153, respectively (1990). The pH value of each treatment was determined according to AOAC 943.02 (1990) on the processing day.

4.3.5 Meat colour analysis

The colour (CIE L* = lightness, a* = redness, b* = yellowness) of fresh burgers were measured using a HunterLab MiniScan XE (Hunter Association Laboratory, Reston, VA) with a 25 mm aperture size according to the method of AMSA (2012). Samples covered with oxygen permeable film were placed to the centre of the probe and colour was read twice by rotating 90 degree. Illuminant A and a 10° observer were used, with the instrument standardized with black

and white tiles. Colour difference (ΔE) was calculated based on the difference of L^* , a^* and b^* values on day 0 and day 3 using the equation as shown below (AMSA, 2012):

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

4.3.6 Myoglobin redox states analysis

Myoglobin redox states were determined according to the method of AMSA (2012) and Mancini et al. (2003). Reference raw meat samples were obtained before determinations on the processing day. Oxymyoglobin reference samples were obtained by placing freshly prepared beef patties in a sealed bag containing 100% oxygen and blooming for 30 min at 4 °C.

Deoxymyoglobin reference samples were obtained by vacuum packaging ground beef and storing at 1 °C for 12 h. Metmyoglobin reference samples were obtained by letting ground beef fully oxidize at 4 °C for 7 days. Burger sample preparation for determining myoglobin redox states was the same as for determination of burger colour. A HunterLab MiniScan XE colorimeter was used. Two readings were made on each sample burger by rotating 90 degree. K/S values (where K = absorbance coefficient, S = scattering coefficient) were measured between 500 to 610 nm in 10 nm intervals. A white ceramic plate was used as reference. Myoglobin redox states of each burger sample were calculated using equations as follows. (Note that since MiniScan only recorded values every 10 nm, values at 470, 520 and 570 nm were used instead of 474, 525 and 572 nm due to the limitation of the equipment).

$$\% \text{Oxymyoglobin} = \frac{\frac{K/S_{610}}{K/S_{525}} \text{ for } 100\% \text{ MMb} - \frac{K/S_{610}}{K/S_{525}} \text{ for sample}}{\frac{K/S_{610}}{K/S_{525}} \text{ for } 100\% \text{ MMb} - \frac{K/S_{610}}{K/S_{525}} \text{ for } 100\% \text{ OMb}} \times 100$$

$$\% \text{Metmyoglobin} = \frac{\frac{K/S_{572}}{K/S_{525}} \text{ for } 100\% \text{ DMb} - \frac{K/S_{572}}{K/S_{525}} \text{ for sample}}{\frac{K/S_{572}}{K/S_{525}} \text{ for } 100\% \text{ DMb} - \frac{K/S_{572}}{K/S_{525}} \text{ for } 100\% \text{ MMb}} \times 100$$

$$\% \text{Deoxymyoglobin} = \frac{\frac{K/S_{474}}{K/S_{525}} \text{ for } 100\% \text{ OMb} - \frac{K/S_{474}}{K/S_{525}} \text{ for sample}}{\frac{K/S_{474}}{K/S_{525}} \text{ for } 100\% \text{ OMb} - \frac{K/S_{474}}{K/S_{525}} \text{ for } 100\% \text{ DMb}} \times 100$$

4.3.7 Thiobarbituric acid reactive substances (TBARS) analysis

TBARS was assayed for each burger sample obtained on day 0, 1, 3, 5, and 7 of refrigerated storage, according to the method of Witte, Krause, & Bailey (1970) and Bedinghaus and Ockerman (1995). Samples taken on Day 0 and Day 1 were wrapped in aluminum foil and stored at -70 °C. These samples were analyzed within 24 h after sampling. Samples taken on Day 3 to Day 7 were analyzed on the same day. One randomly selected piece (1/8 wedge of a burger) from two burgers from each treatment was cut on each assay day and the burger was re-packaged. Five g of each sample was placed in Stomacher sampling filter bag and blended for 60 s using a Stomacher lab blender 400. Fifty mL of TCA solution (20% TCA containing 1.6% phosphoric acid) and 50 mL of cold distilled water was added into the bag and blended for 3 min. The mixture was filtered through Whatman No.1 filter paper. The volume was brought up with 50% TCA: 50% distilled water. Five mL of filtrate was transferred into centrifuging tubes and 5 mL of 0.02 mol/L of TBA was added. The tubes were placed in a boiling water bath for 35 min and then were cooled. The absorbance of the filtrate was measured at 532 nm against a blank. The result (mg malondialdehyde equivalents/kg meat) was calculated from a standard curve prepared from a series of solutions of 1, 1, 3, 3,-tetramethoxypropane with different concentrations.

4.3.8 Statistical analysis

The means and standard deviations (SD) of the three replicates of ten samples (including 2 controls, 2 references, 6 treatments) were calculated. The effects of treatments, storage time and their interactions on the results of colour, myoglobin and TBARS were compared by analysis of variance (ANOVA) using the MIXED procedure of the SAS 9.4 program (SAS Institute, USA). Tukey test procedure was used to do multiple comparisons between individual treatments. Correlation analysis among variables was conducted using CORR procedure. The level of significance was set at $P < 0.05$. The effects of independent variables and their interactions on dependent variables are shown in section 9.2.

4.4 Results and Discussion

4.4.1 Compositional analysis and pH

The results of compositional parameters and pH of each burger treatment are shown in Table 4.2. For the meat block that was used to make burgers, it contained 61.2% moisture, 0.9% ash, 17.6% protein and 19.7% fat with a pH of 5.5. The moisture content of burger samples ranged from 60.3 to 66.5%. This is because there are different levels of ice water added into the burgers, from 8.1 and 8.7 to 13.5 and 14.1% as shown in Table 4.1. Higher ice water level may have caused higher moisture content in the final product. The moisture content in lentil flour was approximately at 10% level, so the incorporation of lentil flour may decrease the moisture content in the final burger product. The protein content of burgers ranged from 14.6 to 16.4%. Since red lentil contains 25% protein (dry weight basis) as shown in study 1, significantly higher ($P<0.01$) protein content is found in treatments with 6% whole seed flour and 5.4% cotyledon flour incorporation compared with control 1, but not higher than control 2 due to its higher meat content. The fat content of burger products ranged from 16.3 to 18.5%, matching the target fat content of 17%. The lower fat content comes from the burgers containing 0.6% lentil seed coat flour or 0.05% sodium erythorbate. This is because lentil contains very low fat, ranging from 0.7 to 3.8% (Bhatty, 1988) and in this particular lentil seed 1.2 % (Study 1). The range in fat level could make a difference on beef burger colour when it was between 10 to 15%, but no difference was found when fat level was between 15 to 25% (Jeong, Lim, & Kim, 2016). The ash content of burgers ranged from 1.6 to 1.7%. The pH of the products ranged from 5.5 to 5.6, with an average pH value of 5.54. Among experimental samples, burgers with 6.0% added whole seed flour and 5.4% cotyledon flour are quite identical in moisture, ash, protein, fat content and pH. Since a higher amount of cotyledon (5.4%) was added into the burgers than seed coat (0.6%) and the margin was replaced using water, lower moisture, higher ash, protein and fat content can be expected in the burgers incorporated with cotyledon flour than those with added seed coat flour.

Table 4.2 Composition analysis of all beef burger samples (Mean \pm SD)

Treatment^{1,2}	Moisture (%)	Ash (%)	Protein (%)	Fat (%)	pH
Control 1-low binder equivalent	65.5 \pm 0.8	1.6 \pm 0.1	14.6 \pm 0.6	17.7 \pm 0.3	5.5 \pm 0.0
Control 2-high binder equivalent	63.3 \pm 0.3**	1.7 \pm 0.1	16.1 \pm 0.1**	18.5 \pm 0.1	5.5 \pm 0.0
Toasted wheat crumb (TWC), 6.0%	60.3 \pm 0.7**	1.6 \pm 0.0	15.4 \pm 0.4*	17.4 \pm 0.9	5.5 \pm 0.1
Sodium erythorbate, 0.05%	66.5 \pm 0.2	1.6 \pm 0.0	15.0 \pm 0.2	16.5 \pm 0.5*	5.5 \pm 0.0
Raw lentil, whole seed, 6.0%	60.5 \pm 0.2**	1.7 \pm 0.1*	15.9 \pm 0.0**	17.4 \pm 0.6	5.6 \pm 0.0**
Raw lentil, cotyledon, 5.4%	60.8 \pm 0.5**	1.7 \pm 0.0*	16.1 \pm 0.3**	17.6 \pm 0.5	5.6 \pm 0.1**
Raw lentil, seed coat, 0.6%	65.8 \pm 0.7	1.6 \pm 0.0	15.0 \pm 0.4	16.3 \pm 0.8*	5.5 \pm 0.1
Heat-treated lentil, whole seed, 6.0%	60.3 \pm 0.7**	1.7 \pm 0.1	16.3 \pm 0.5**	17.3 \pm 0.7	5.6 \pm 0.0**
Heat-treated lentil, cotyledon, 5.4%	61.1 \pm 0.9**	1.7 \pm 0.0*	16.4 \pm 0.5**	16.8 \pm 0.6	5.6 \pm 0.1**
Heat-treated lentil, seed coat, 0.6%	65.4 \pm 0.4	1.6 \pm 0.0	15.0 \pm 0.8	16.8 \pm 0.4*	5.5 \pm 0.0

¹N=3.²Means with ** and * are significantly different (P<0.01, P<0.05, respectively) from Control 1.

4.4.2 Meat colour

The a* value represents the redness of a raw beef burger and consumers expect the bright red colour of raw meat products (Carpenter et al., 2001). The changes of a* value of all samples during storage are shown in Figure 4.1. On day 0, burgers with 6% whole seed flour of both raw and heat-treated lentil showed significantly lower a* values (P<0.05) than the controls (Figure 4.1). This is probably because the lentil flour may transfer its own colour to beef burgers. It is found that the a* value of lentil flour of red lentil (whole seed) are 6.3 for raw and 10.8 for heat-treated one, while the a* value of raw beef meat is around 32 as observed from the control sample. Such colour transferring effect from non-meat ingredients was also reported by others; for instance, green leaf extracts may bring their greenish colour to meat products and affect the redness as a result (Kim et al., 2013).

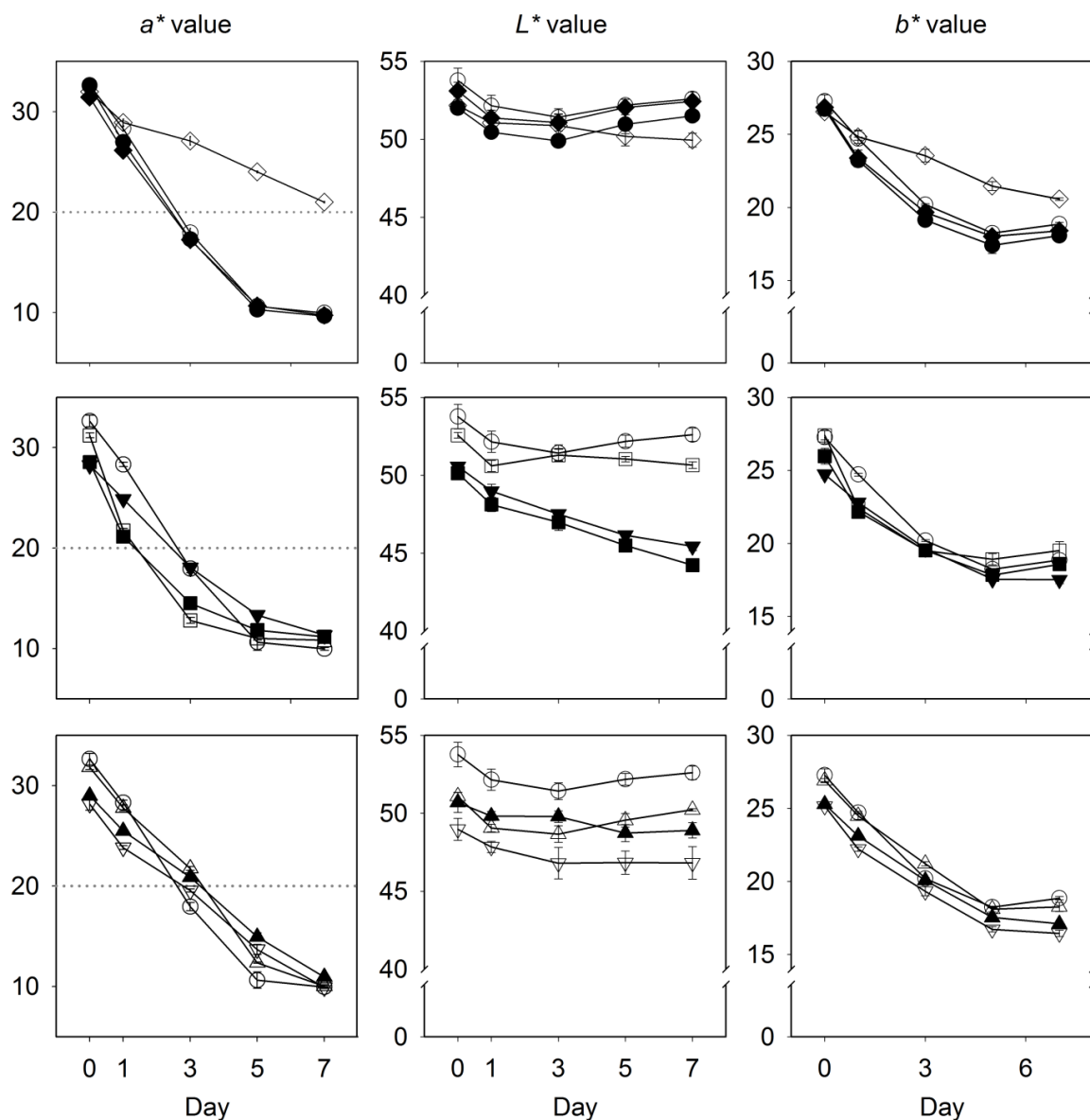


Figure 4.1 Colour changes (a*, L* and b* values) of all beef burger samples stored at 4 °C for 7 days. ○ Contol 1, ● Control 2, ◇ Sodium erythorbate-0.05%, ◆ Toasted wheat crumb-6.0%, □ Raw lentil, cotyledon-5.4%, ■ Raw lentil, whole seed-6.0%, △ Heat-treated lentil, cotyledon-5.4%, ▲ Heat-treated lentil, seed coat-0.6%, ▽ Heat-treated lentil, whole seed-6.0%, ▼ Raw lentil, seed coat-0.6%

During the 7 storage at 4 °C, a* value of all treatments shared a decreasing trend, but the degree of such decrease varied from treatment to treatment, especially from day 0 to day 3 (Figure 4.1). After day 3, a* value of most treatments dropped very rapidly. As a result, day 3 can be seen as a cut off (Table 4.3). When the a* value of the beef burger has decreased below 20, the surface colour is not considered as red, but brown (Poulson, 2015). On day 3, burgers of control 1 and 2 had a* values around 17.3 - 17.9. Reference burgers with 6% TWC showed a similar a* value at 17.2 and did not differ ($P>0.05$) from the control 1. However, the burgers with 0.05% sodium erythorbate displayed the highest a* value (27.1) of all treatments (Table 4.3). This can be attributed to the potent antioxidant effect of sodium erythorbate and such effect was also found in raw ground beef by other researchers (Suman et al., 2005; Sepe et al., 2005).

Table 4.3 Redness (a* value) on day 3 and colour difference (ΔE) from day 0 to day 3 of beef burger treatments

Treatment	a* value on day 3	ΔE (day 0 to day 3)
Control 1-low binder equivalent	17.9 ± 0.4^{cde}	16.5 ± 5.1^a
Control 2-high binder equivalent	17.3 ± 0.3^{de}	17.2 ± 4.2^a
Toasted wheat crumb (TWC), 6.0%	17.2 ± 0.3^{de}	16.1 ± 2.8^{ab}
Sodium erythorbate, 0.05%	27.1 ± 0.4^a	6.0 ± 1.4^d
Raw lentil, whole seed, 6.0%	14.5 ± 0.2^{ef}	15.8 ± 1.5^{ab}
Raw lentil, cotyledon, 5.4%	12.8 ± 0.3^f	20.1 ± 2.4^a
Raw lentil, seed coat, 0.6%	18.1 ± 0.1^{cde}	11.9 ± 1.0^{bc}
Heat-treated lentil, whole seed, 6.0%	19.5 ± 0.2^{bcd}	10.7 ± 0.7^c
Heat-treated lentil, cotyledon, 5.4%	21.7 ± 0.2^b	11.8 ± 2.1^{bc}
Heat-treated lentil, seed coat, 0.6%	20.9 ± 0.2^{bc}	9.7 ± 1.7^{cd}

¹N=3

²Means with different superscripts within each column are significantly different ($P<0.05$)

Addition of raw or heat-treated lentil flours to beef burgers showed contrasting effects on burger colour on day 3. Burgers with 6% raw lentil flour displayed lower a* value than the

controls while burgers with 6% heated lentil flour showed higher a^* value than the controls. The difference between these two experimental samples was statistically significant ($P < 0.05$) on day 3 (Table 4.3). The negative effect of raw lentil on burger colour from heat-treated lentil flour could be explained from results of Study 1 that demonstrated high activity of oxidative enzymes, such as lipoxygenase and peroxidase in raw lentil cotyledon (see section 3.4.2.1 and 3.4.3.2). These oxidative enzymes could contribute to faster lipid oxidation which resulted in higher amount of radicals and more oxidative stress (Baysal & Demirdöven, 2007; Burnette, 1977). Since the products of lipid oxidation could induce the oxidation of oxymyoglobin (Lynch & Faustman, 2000), the higher amount of these products, such as 4-HNE could lead to a higher level of metmyoglobin and change the predominant colour to brown. After effective heat treatment, such as 150 - 165 °C of infrared heating or 90 °C water bath heating for 30 min, these oxidative enzymes can be deactivated (Pathiratne et al., 2015; see section 3.4.2.1). Thus, no adverse effect on surface colour was found for burgers with added heat-treated lentil flour. A lentil flour mixture of half raw and half heat-treated seed was also evaluated as a binder added at 6% to beef burgers (see Appendix section 9.2). This sample would have half the lipoxygenase activity as the raw seed flour. The results indicated that even the addition of half-half flour (raw:heat = 50:50) had the same adverse effect on redness of beef burgers as the raw lentil flour, indicating the strong negative effect of lipoxygenase on meat colour.

On the other hand, heat-treated lentil flour (whole seed) has the capability to slow down the decrease in red colour in the beef burgers, as the a^* value was higher than the controls on day 3 (Table 4.3). The enhanced colour stability may be resulting from the high antioxidant activity contributed by high content of phenolic compounds (Han & Baik, 2008) and from heat stable antioxidative enzymes (see section 3.4.2.1 and section 3.4.2.2). Heat treatment has been shown to decrease the amount of phenolic compounds in grains and legumes, but the antioxidant potential can remain due to the development of new forms of compounds (Nayak et al., 2015). From our observations, antioxidant activity contributed by soluble components of lentil and superoxide

dismutase (SOD) activity were not affected by heat treatment (see section 3.4.2.1). As a result, these antioxidants could scavenge radicals and protect meat colour from faster browning.

Among the experimental samples with added heat-treated lentil flour, the a^* value of burgers with 0.6% added seed coat flour did not differ ($P>0.05$) from those with 5.4% cotyledon flour, despite the large differences in phenolic content of the flours. This suggests that both seed coat and cotyledon exhibited strong antioxidant ability to protect colour and would be effective binders. The effectiveness of cotyledon flour may be due to its higher addition level than seed coat (5.4% versus 0.6%). In fact, from the results of the previous study (see section 3.4.5), cotyledon provides comparable total water soluble phenolic content as seed coat if their natural proportion in seeds is considered. Also, a sharp increase in ferrous ion chelating ability was observed for the soluble proteins extracted from cotyledon after heat treatment (see section 3.4.4.5), and this may contribute to the colour protection ability of the heat-treated cotyledon flour in beef burgers. Compared to the burgers with added raw lentil flour, the seed coat incorporated burgers had improved ($P<0.05$) a^* value compared with the incorporation of cotyledon flour. On day 3, the a^* value of samples with added raw cotyledon flour dropped to 12.8 while the a^* value for samples with added raw seed coat flour remained at 18.1 ($P<0.05$). This is because the oxidative enzymes are mainly located in the cotyledon part while seed coat contributes more antioxidant activity (see section 3.4.2.1). In fact, the content of phenolic compounds in lentil seed coat (hull) is seven or eight times higher than that in whole seed (Oomah et al., 2011). From the results shown in the previous study (see section 3.4.5), total phenolic content of seed coat was at least six times higher than that of cotyledon. The a^* value of burgers with added raw whole seed flour was 14.5 on day 3, in between those with added raw flour of seed coat (18.1) and cotyledon (12.8), possibly due to the presence of both antioxidative and oxidative components. There are also reports on the binding capacity of phenolic compounds to deactivate enzymes, such as lipoxygenase and peroxidase (Laughton, Evans, Moroney, Hault,

& Halliwell, 1991; Baynton et al., 1994). The highest phenolic content was found in the raw whole seed flour, which may have moderated the activity of the oxidative enzymes.

L* value represents the lightness of the surface of beef burgers as shown in Figure 4.1. On day 0, the highest L* value is 53.8 from the Control 1, while the lowest L* value is 49.0 of the burger samples with heat-treated whole seed flour at 6%. The burgers samples containing lentil seed coat flour, no matter whether as whole seed flour (raw and heat-treated) or seed coat itself (raw and heat-treated), were significantly ($P<0.05$) darker in colour compared with the Control 1. This is probably because their incorporation brings the dark colour of lentil seed coat (with L* value of 48.6 - 51.2) flour into the burgers. The L* value of the seed coat flour (48.6 - 51.2) used in this study is much darker ($P<0.05$) than that of the cotyledon flour (86.5 - 86.9). Darkening of ground pork also occurred when leaf extracts of curry and mint were added (Biswas, Chatli, & Sahoo, 2012). However, the incorporation of TWC or raw lentil cotyledon flour did not show any effect on lightness ($P>0.05$) compared with the controls.

Throughout the 7 day storage, b* value (yellowness) of all samples decreased ($P<0.001$) as shown in Figure 4.1. The addition of whole seed lentil flour into beef burgers did not show any significant effect ($P>0.05$) on b* values of burgers. However, the incorporation of different physical components of lentil seed, namely seed coat and cotyledons, contributed different yellowness to beef burgers, that was the burger with added cotyledon flour showed relatively higher ($P<0.01$) b* value than the burgers with added seed coat flour. This could be explained by the colour contribution of the flours themselves (the b* value of seed coat was lower than that of cotyledon (14.25 - 18.41 and 24.92 - 27.09, respectively)).

Colour difference (ΔE) of all treatments from day 0 to day 3 is shown in Table 4.3. ΔE integrates all three parameters (L*, a* and b* values) into one parameter. From day 0 to 3, the controls showed higher ($P<0.05$) colour difference than burgers with added heat-treated lentil flour (whole seed), but did not differ from burgers with added raw lentil flour (whole seed).

Burgers with added raw cotyledon flour ΔE value of 20.1, indicating that large colour changes took place during these three days. In contrast, burgers with added raw seed coat flour showed lower ($P<0.05$) colour changes (ΔE) than that of burgers with added raw cotyledon flour during the 3 day storage. Burgers with 6% toasted wheat crumb had a similar ($P>0.05$) colour difference as the controls, but burgers with 0.05% sodium erythorbate showed lowest colour change of all samples. Hence, the addition of heat-treated lentil flour into beef burgers (ΔE at 10.7) should be considered as a benefit to protect colour from major changes during storage. Traditional binders, such as wheat flour or soybean proteins did not show a colour protection effect for fresh beef burgers (Wanasundara, & Pegg, 2007) in contrast to that observed in the present study following incorporation of heated lentil flour.

4.4.3 Myoglobin redox states

Myoglobin redox states of each treatment during storage are shown in Figure 4.2. On day 0, the oxymyoglobin form dominated the myoglobin form of the burger surface of all treatments with much higher level (78.4 - 95.2%) than metmyoglobin (0.0 - 15.0%) and deoxymyoglobin (0.9 - 16.7%). During storage, the deoxymyoglobin and oxymyoglobin on the surface of burgers decreased while metmyoglobin increased. This is because the oxymyoglobin was gradually oxidized to form metmyoglobin (Mancini & Hunt, 2005), especially since we used oxygen permeable film.

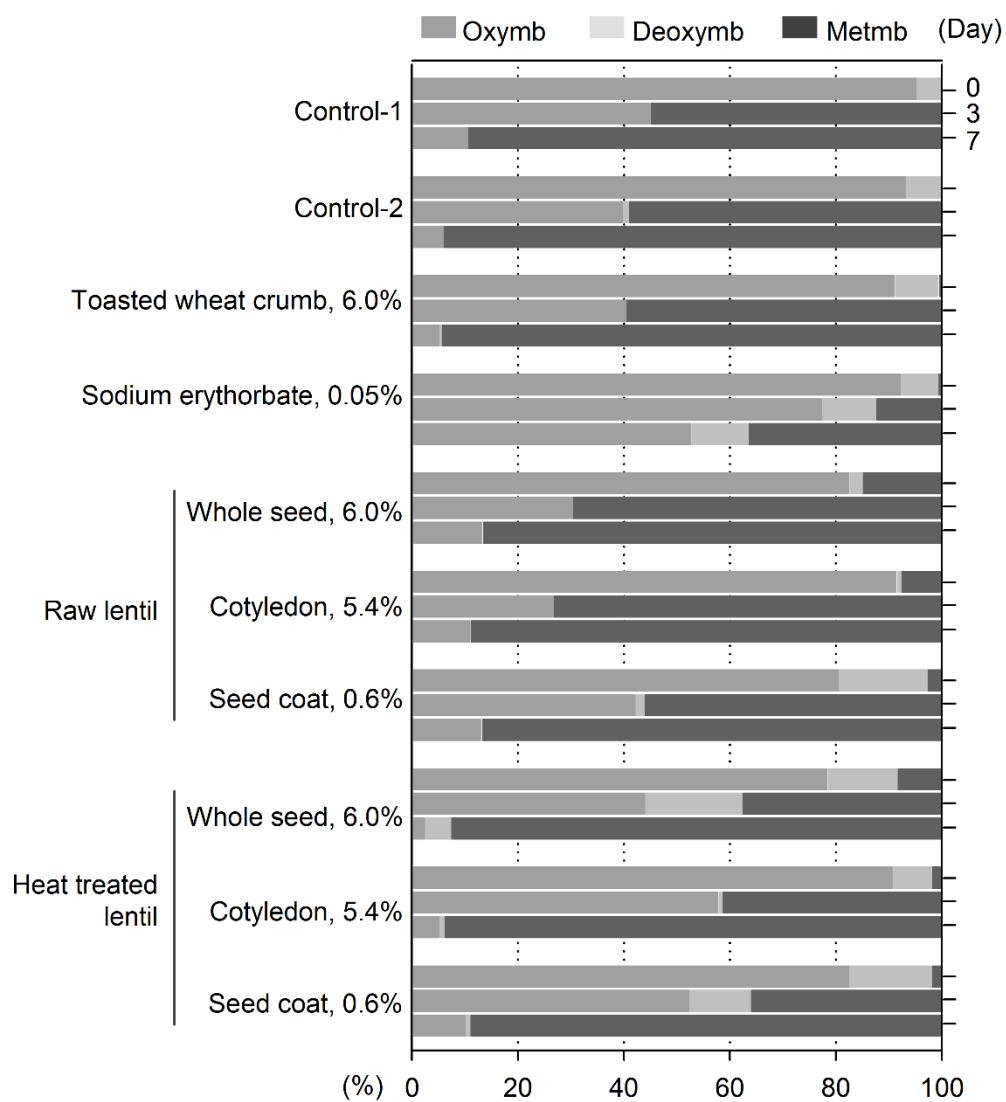


Figure 4.2 Myoglobin redox states of different beef burger samples during refrigerated storage for 7 days.

According to the surface colour parameters of burgers, the redness of each treatment showed the greatest difference on day 3. On that day, burger samples with added heat-treated flour (whole seed) also showed lower ($P<0.05$) metmyoglobin level (37.7%) than the controls (55.0 - 59.0%) and the burgers with added raw seed flour (whole seed) (69.6%). Accordingly, the oxymyoglobin level of the burgers with added heat-treated flour were generally higher ($P<0.05$) than the controls and the burgers with added raw seed flour on day 3. A previous study reported that adding raw chickpea flour could increase the metmyoglobin formation in meat batters due to the lipoxygenase activity (Verma, Ledward, & Lawrie, 1984). However, heating of chickpea flour at 80 °C for 1 hour, the ability to elevate metmyoglobin level was decreased. We previously found that lipoxygenase in the lentil significantly decreased after infrared heat treatment to a seed surface temperature of 150 °C (see section 3.4.2.1; Pathiratne et al., 2015). Such heat treatment was also effective in lowering the metmyoglobin formation in beef burgers during storage. There was no difference ($P>0.05$) found on metmyoglobin level among the burgers with added heat-treated flours (whole seed 37.7%, cotyledon 41.4% and seed coat 36.0%). The low level of metmyoglobin in burgers with added seed coat should be owing to the high level of phenolic compounds. The addition of heat-treated cotyledon flour also lowered the level of metmyoglobin of burgers. One reason could be the deactivation of oxidative enzymes in lentil by heat treatment as shown in section 3.4.2.1. The other reason should be the elevated ferrous ion chelating ability of soluble proteins by heat treatment as shown in section 3.4.4.5. Researchers have found that metal chelators (type II antioxidants) have a stronger ability to protect oxymyoglobin from being oxidized to metmyoglobin than radical scavengers (type I antioxidants) (Allen & Cornforth, 2009).

The metmyoglobin level of burgers with added raw seed coat flour were generally lower ($P<0.01$) than those with added raw cotyledon flour. This also should be attributed to the nearly zero activity of oxidative enzymes and higher level of phenolic compounds present in lentil coat than in seed cotyledon as we observed from the previous study (see section 3.4). The reason that

phenolic compounds can maintain higher amount of oxymyoglobin could be because of the ability of these antioxidants to reduce Fe^{3+} to Fe^{2+} . Researchers built a model system containing metmyoglobin and various phenolic compounds, and found that a higher concentration of phenolic compounds can help to reduce metmyoglobin to form oxymyoglobin (Miura et al., 2014). Due to the reducing ability of phenolic compounds, metmyoglobin could be converted back to oxymyoglobin and in this way, the level of metmyoglobin can remain low. We also found a similar effect on metmyoglobin due to lentil seed coat extracts in the previous study (see section 3.3.6), approx. 20% of metmyoglobin in the model system can be reduced to form oxymyoglobin with the presence of seed coat extracts, while seed cotyledon extracts did not show such strong reducing ability.

Sodium erythorbate added to beef burgers at 0.05% successfully maintained the oxymyoglobin level (>50%) throughout the 7 days of retail display, as also demonstrated by the higher a^* values of the burger surface as shown in Figure 4.2. Sodium erythorbate is a common antioxidant used in food applications (Barringer, Abu-Ali, & Chung, 2005). From the results of day 3, burgers with added sodium erythorbate had the highest oxymyoglobin level and the lowest metmyoglobin level of all treatments. Other researchers also found that 500 ppm (0.05%) ascorbic acid added to beef patties maintained red colour with a lower metmyoglobin level than that of the control patty during storage at 2 °C for 20 days (Sánchez-Escalante, Djenane, Torrescano, Beltrán, & Roncalés, 2001). This is because ascorbic acid can induce a reduction of metmyoglobin to form oxymyoglobin (Tsukahara & Yamamoto, 1983). TWC added into beef burgers as a binder at 6% did not perform similarly as the heat-treated lentil flour. The a^* value and oxymyoglobin level of the burgers with added TWC were lower ($P < 0.05$) than burgers with added heat-treated lentil flour on day 3. This suggested that heat-treated lentil could be potential binder with the added benefit of increasing colour stability compared with the traditional binder.

4.4.4 TBARS

The TBARS assay measures the secondary reaction products of lipid oxidation, mainly malondialdehyde in samples (St. Angelo, Vercellotti, Jacks, & Legendre, 1996). TBARS values of all treatments during 7 day storage are shown in Figure 4.3. On day 0, all samples had low TBARS values ranging from 0.74 to 1.14 mg MDA/kg sample. However, the TBARS values of controls increased rapidly within seven days and were significantly higher than other treatments on day 7. TBARS values of all treatments started to show differences on day 3 and on day 7, the largest difference among treatments was observed. Similar results were reported by Sánchez-Escalante, Djenane, Torrescano, Beltrán and Roncales (2003) using borage (1% and 2%), rosemary (0.1%) and oregano (0.02% and 1%) in beef patties. In their results, greatest colour difference among treatments was observed on day 12 while the largest TBARS difference was found on day 16. This could possibly be because TBARS value is a measurement of secondary products of lipid oxidations (St. Angelo et al., 1996). There are primary products of lipid oxidation, such as peroxides and free radicals which developed ahead of these secondary products (Kerrihard, Pegg, Sarkar, & Craft, 2015).

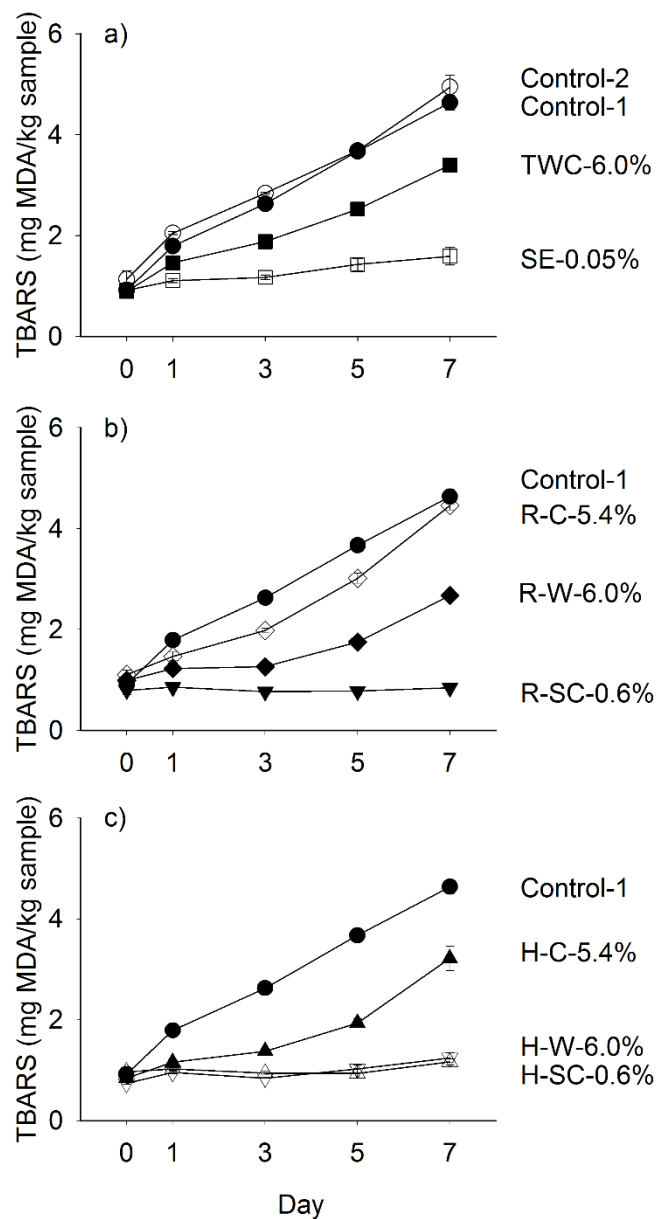


Figure 4.3 TBARS values of all beef burger samples stored at 4 °C for 7 days, a) controls and reference samples, TWC - Toasted wheat crumb, SE - Sodium erythorbate; b) Samples with raw lentil seeds, R - Raw lentil, C - Cotyledon, W - Whole seed, SC - Seed coat; c) Samples with heat-treated lentil seeds, H - Heat-treated lentil.

On day 7, TBARS of burgers with added heat-treated lentil flour (1.25 mg MDA/kg sample) was significantly lower ($P<0.05$) than those with added raw lentil flour (2.68 mg MDA/kg sample) and controls (4.63 - 4.94 mg MDA/kg sample). Burgers with added raw cotyledon flour showed comparable TBARS values as the control burgers, while burgers with added heat-treated cotyledon flour had lower ($P<0.05$) TBARS values. The difference between burgers with raw and heat-treated lentil again highlighted deleterious effects of oxidative enzymes, such as lipoxygenase promoting lipid oxidation in meat products. Lipoxygenase has been proven to initiate lipid oxidation in food products by catalyzing the unsaturated fatty acids containing *cis,cis*-1,4-pentadiene and causes off-flavour and more rapid lipid oxidation as a result (Eriksson, 1982). No matter whether the lipoxygenase is endogenous (German, Chen, & Kinsella, 1985) or exogenous (King, Chin, Svendsen, Reitmeier, Johnson, & Fehr, 2001), it initiates and accelerates lipid oxidation and causes the deterioration of meat products by producing off-flavours. Also, an elevated TBARS was observed for fish mince with the presence of lipoxygenase compared to the control (Fu, Xu, & Wang, 2009). The heat treatment applied to lentil seeds deactivated the activity of oxidative enzymes present in lentil and therefore, decreased the lipid oxidation either in lentil itself or in meat systems as shown by the lower TBARS values in the burgers with added heat-treated lentil flour.

Burgers with added flour from seed coat or whole seed generally showed significantly ($P<0.05$) lower TBARS values than burgers with added cotyledon flour. For burgers with added heat-treated lentil flour, the difference between those with added seed coat (1.16 mg MDA/kg sample) and cotyledon (3.21 mg MDA/kg sample) became significant different on day 7 ($P<0.05$). For the burgers with added raw lentil components, the difference between those with added seed coat (0.77 mg MDA/kg sample) and cotyledon (1.98 mg MDA/kg sample) showed up starting from day 3 ($P<0.05$). The different performance of the seed coat and cotyledons on the TBARS values of beef burgers could be explained by the high amount of phenolic compounds in lentil seed coat. Phenolic compounds such as flavonoids, phenolic acids and tannins have

antioxidant properties which contribute to the nutritional importance of lentil (Amarowicz & Pegg, 2008). More importantly, although the weight proportion of seed coat is much lower (8.2 - 11.4%) than that of cotyledon (88.6 - 91.8%), catechins, procyanidins, flavonols and flavones in seed coat are the main contributors to the total phenolic content of lentil, whereas cotyledons only provide a small proportion of phenolic compounds, mainly cinnamic and benzoic compounds (Dueñas et al., 2002). This could also be the reason that burgers with added raw cotyledon flour displayed higher ($P<0.05$) TBARS values than burgers with added raw whole seed flour on day 7.

The oxidation of lipids and myoglobin are interrelated. In Study 1 (see section 3.3.7), it was shown that metmyoglobin can accelerate the oxidation rate of linoleic acid by 1000 fold in the model system. Adding the extracts from lentil seed coat can dramatically decrease the oxidation rate initiated by metmyoglobin while the extracts of cotyledon cannot. This also indicates the higher antioxidant ability to prevent lipid oxidation of lentil seed coat than cotyledon.

Among the reference samples, burgers with 6.0% TWC had lower ($P<0.05$) TBARS values than the control, but higher values ($P<0.05$) than burgers with added seed coat flour or heat-treated whole seed flour. Burgers with the seed coat components or whole flour showed similar low TBARS values as those with 0.05% SE, indicating that they all provided potent antioxidant activity. Wheat flour also contains phenolic compounds at approx. 0.7 - 0.9 mg ferulic acid eq./g defatted sample that exert antioxidant ability to inhibit lipid oxidation to some extent (Liyana-Pathirana, & Shahidi, 2006).

There are a number of reports on the effect of antioxidants on colour protection ability and delay of lipid oxidation in raw meat products. These antioxidants could come from commercial sources, such as tea catechins (Mitsumoto et al., 2005) and grape seed extracts (Bañón et al., 2007). They could also be extracts from natural herbal sources, such as extracts from peanut skin (Yu et al., 2010), willowherb (Cando et al., 2014) or vegetable leaf (Kim et al., 2013). All of these provide antioxidant effects by slowing down lipid oxidation in raw meat products. However, the

benefit of lentil flour is that it can also serve as a binder in meat products and assist in increasing the protein content and elevating water holding capacity due to the comparatively high levels of protein and carbohydrates (Der, 2010). Meanwhile, heat-treated lentil flour showed some advantages compared with the traditional binder since it protected meat colour and delayed lipid oxidation.

4.4.5 Correlation analysis

Correlation analysis among instrumental colour measurements, myoglobin species and TBARS value was carried out for refrigerated stored samples (Table 4.4) and found that all the correlation coefficients among variables were significant. For the refrigeration storage, the redness (a^* value) of the samples, was found to be highly correlated with yellowness (b^* value), oxymyoglobin level and deoxymyoglobin level ($r = 0.94, 0.87$ and 0.90 , respectively). All these values were low during storage compared to zero time. The a^* value showed a negative correlation with metmyoglobin level ($r = -0.98$) and TBARS value ($r = -0.62$) of the samples. The high correlation between a^* value and metmyoglobin is because the accumulation of metmyoglobin is the cause of the decrease of a^* value of the surface of beef burgers. The lower correlation between a^* value and TBARS value is because the development of TBARS was slower than the colour changes associated with the formation of metmyoglobin. The metmyoglobin level is correlated with TBARS value ($r = 0.66$), but negatively correlated with oxymyoglobin and deoxymyoglobin ($r = -0.92$ and -0.89 , respectively). The decrease in oxymyoglobin and deoxymyoglobin occurred during storage as they were oxidized to metmyoglobin.

Allen and Cornforth (2010) have found a positive correlation between a^* value and oxymyoglobin level ($r = 0.72, P < 0.001$) and a negative correlation between a^* value and TBARS value ($r = -0.44, P < 0.001$) for fresh beef samples. A negative correlation between a^* value and

metmyoglobin ($r = -0.89$, $P < 0.01$) was also observed for raw beef patties during storage (Liu et al., 2015).

Table 4.4 Correlation coefficients among dependent variables measured on beef burgers during refrigerated storage

	a*	L*	b*	MetMb	OxyMb	DeoxyMb
L*	0.33***					
b*	0.94***	0.47***				
MetMb	-0.98***	-0.25***	-0.86***			
OxyMb	0.87***	0.33***	0.72***	-0.92***		
DeoxyMb	0.90***	0.88***	0.88***	-0.89***	0.66***	
TBARS value	-0.62***	-0.44***	-0.44***	0.66***	-0.58***	-0.59***

Note: *** refers to significant correlation ($P < 0.001$)

4.5 Conclusions

Adding heat-treated lentil flour can successfully slow down the reduction of redness (a^* value), formation of metmyoglobin and progression of unsaturated lipid oxidation during the refrigerated storage of beef burgers when compared with the control burgers. However, the raw lentil flour (whole seed) did not perform as effectively as those heat-treated ones, possibly because of the presence of oxidative enzymes as confirmed by the results of Study 1. The seed coat components when tested in the natural proportion of seed coat in the flour showed high antioxidant activity in protecting colour and slowing down lipid oxidation of raw beef burgers regardless of heat treatment, possibly because it contains high level of phenolic compounds (and was low in oxidative enzymes). However, cotyledon flour from heat-treated or raw seeds showed opposing effect on colour changes and lipid oxidation. Due to the inactivation of oxidative enzymes, heat-treated cotyledon flour was able to provide an antioxidative effect that can protect fresh colour of meat and also can inhibit lipid oxidation. Therefore, a thermal treatment is a necessary pre-heating step for whole lentil or lentil cotyledon to eliminate activity of oxidative

enzymes and this definitely helps in protecting fresh meat colour and delaying lipid oxidation when lentil flour is incorporated in fresh, ground meat products.

4.6 Connection to the next chapter

Refrigerated storage at 4 °C is a common storage condition for raw meat products. The frozen storage is also used for these type of meat products to extend their shelf life and help in product transportation and distribution. At -20 °C, the effect of lentil flour could be different than observed under refrigeration. For example, the oxidative enzymes may not work at such a low temperature, and it is not known whether phenolic compounds would work as antioxidants to protect colour and to prevent lipid oxidation. The effect of lentil flour on the colour and lipid oxidation of beef burgers under frozen storage was evaluated in the following study.

5 STUDY 3 USE OF THE ANTIOXIDANT ABILITY OF LENTIL FLOUR TO PROTECT MEAT COLOUR AND TO DELAY LIPID OXIDATION DURING FROZEN STORAGE OF BEEF BURGERS

5.1 Abstract

The oxidation of myoglobin and unsaturated lipids of raw meat products during frozen storage affects the quality of final product. In this study, beef burgers were incorporated with flour from different seed components, namely the whole seed (at the level of 6.0%), cotyledon (at 5.4%) and seed coat (at 0.6%) of raw and heat-treated lentil seeds. The effects of lentil flour on the colour changes, myoglobin redox states and lipid oxidation of beef burgers under frozen storage at -20 °C for 12 weeks were evaluated in three-week intervals. Adding lentil flour from either heat-treated or raw seeds into beef burgers led to higher a^* values, lower metmyoglobin levels and lower Thiobarbituric acid reactive substance (TBARS) values at 6 to 12 weeks of frozen storage compared with the all meat control. Burgers with added heat-treated cotyledon flour showed higher a^* value and lower metmyoglobin level than those with added seed coat flour, with or without heat treatment. No difference was found in burgers with added raw lentil seed components. Lower TBARS values were found for burgers with added heat-treated lentil flour than those with added raw lentil flour at the end of storage. Adding sodium erythorbate at 0.05% caused a purple surface colour of frozen beef burgers due to the high level of deoxymyoglobin formed. Toasted wheat crumb at 6.0% showed some antioxidant effect on colour protection and inhibition of lipid oxidation, but not as strong as the heat-treated lentil flour. Significant negative correlations were found between a^* and metmyoglobin and between a^* and

TBARS values. Adding heat-treated lentil flour can help beef burgers to maintain the surface redness and to inhibit lipid oxidation during frozen storage by exerting antioxidant activity.

5.2 Introduction

Frozen storage is a common way to store meat products since the very low temperature can slow down the physical and biochemical reactions that may lead to the deterioration of foods (George, 1993). As a result, the shelf-life of frozen meat products can be prolonged compared with refrigerated ones (Leygonie et al., 2012). In the United States, the market size of frozen ground beef products (including burgers, meatballs, minced meat and others) was over 2.02 billion dollars in 2013, even higher than the market size of the chilled ground beef products (1.99 billion dollars) (Statistics Canada, 2012b). But during the frozen storage, meat products may still suffer some unfavorable changes of quality, such as the loss of moisture (Añón & Calvelo, 1980), the denaturation of proteins (Wagner & Añón, 1985) and the oxidation of proteins (Soyer, Özalp, Dalmış, & Bilgin, 2010). Also, discoloration and development of lipid oxidation products (TBARS values) were observed for ground beef (Brewer & Wu, 1993) and beef patties (Utrera, Morcuende, & Estévez, 2014) during frozen storage. This may lead to lower consumer acceptance and higher food waste. The oxidation of myoglobin and lipid in raw meat products are believed to be interactive (Faustman et al., 2010), that is the products of lipid oxidation, such as 4-HNE can promote the oxidation of myoglobin (Lynch & Faustman, 2000) while the presence of metmyoglobin can increase the rate of lipid oxidation in the same system (Hayashi, Uchida, Takebe, & Takahashi, 2004).

Researchers have utilized different approaches to prevent the quality loss of meat products induced by myoglobin and lipid oxidation under frozen conditions. Vacuum packaging is one approach that can successfully preserve the colour and can inhibit lipid oxidation in long term frozen storage, compared with oxygen permeable packaging (Brewer & Harbers, 1991; Brewer & Wu, 1993). Another way is to incorporate various natural and synthetic antioxidants into meat

products. For instance, chitosan and rosemary extracts (0.02%) were used to protect the colour of beef patties during frozen storage at -18 °C for 180 days (Georgantelis et al., 2007). In another study, grape seed extracts were added to beef and pork patties at 0.02% and they decreased lipid oxidation during frozen storage at -18 °C for 4 months compared with other samples (Rojas & Brewer, 2008). Antioxidants such as rosemary extract, green tea extract, tocopherol, TroloxTM and ascorbic acid were added to pork patties and all of them were found effective on the inhibition of lipid oxidation during frozen storage at -18 °C for 4 months (Haak, Raes, & De Smet, 2009).

In previous studies conducted by our group, it was found that the flour of lentil seed can be used not only as a binder in meat products, but as a source of antioxidants that can protect colour and inhibit lipid oxidation of meat products under refrigerated conditions (Der, 2010; Pathiratne, 2014; also see section 4.4 of Study 1). Lentil seeds can be physically separated into seed coat and cotyledon. The seed coat is low in protein, but contains more phenolic compounds and superoxide dismutase while the cotyledon contains higher protein and oxidative enzymes, but is low in phenolic compounds (see section 3.4). The main antioxidant capability comes from various types of phenolic compounds located in the outer seed coat and the inner cotyledon (Oomah et al., 2011). However, little is known whether such application can simply protect colour and inhibit lipid oxidation during frozen storage. To achieve better performance, heat treatment such as infrared radiation (IR) can be applied on raw lentil seed to eliminate enzyme activities and to protect seeds from faster lipid oxidation (Satya et al., 2010; Zheng et al., 1998). The raw lentil contains highly active oxidative enzymes including lipoxygenase and peroxidase which may promote lipid oxidation not only in lentil seeds, but in meat products as well (Pathiratne et al., 2015; see section 3.4.2.1). However, the effect of heat treatment on lentil and on the properties of meat products incorporated with lentil flour under frozen storage conditions has not been clearly documented.

As a result, this study was designed to investigate the incorporation of raw and heat-treated lentil flour of different seed components, including whole seed, cotyledon and seed coat, and their effect on colour protection and on prevention of lipid oxidation of beef burgers.

5.3 Materials and Methods

5.3.1 Raw materials and chemicals

The raw materials and chemicals used in this study are same as section 4.3.1.

5.3.2 Heat treatment of lentil

The heat treatments of lentil are same as the procedure described in section 4.3.2.

5.3.3 Beef burger formulation and processing

The formulation and processing of beef burgers are the same as that described in section 4.3.3 of Study 2. In total, four burgers per treatment were made (two for colour and myoglobin measurement and two for TBARS measurement) for each of three replications and loosely covered with wax paper on both sides. Each burger was placed in a plastic bag (low density polyethylene, 12.7 cm × 29.2 cm, Ronco, ON) and stored in corrugated boxes. Newly made samples were put into a -30 °C freezer to conduct blast freezing overnight and then were transferred to a -20 °C freezer and stored for 12 weeks. Surface colour, myoglobin and TBARS analysis were conducted on week 0, 3, 6, 9 and 12.

5.3.4 Meat colour analysis

The colour (CIE L* = lightness, a* = redness, b* = yellowness) of frozen burgers were measured using a HunterLab MiniScan XE (Hunter Association Laboratory, Reston, VA) according to the method of AMSA (2012). Frozen samples were taken out from the freezer and the surface of each burger was warmed up by hand over the wax paper to eliminate ice crystals. The wax paper was then removed and a layer of oxygen permeable film was temporarily placed on the surface of the burger and the colour measurements were done within 5 min. Samples were

placed to the centre of the probe and were read twice by rotating 90 degrees. Illuminant A and a 10° observer were used, with the instrument standardized with black and white tiles.

5.3.5 Myoglobin redox states analysis

Myoglobin redox states of each sample was conducted according to the procedure described in section 4.3.6 of Study 2.

5.3.6 Thiobarbituric acid reactive substances (TBARS) analysis

Thiobarbituric acid reactive substances analysis of each sample was conducted according to the procedure described in section 4.3.7 of Study 2.

5.3.7 Statistical analysis

The means and standard deviations (SD) of ten samples (including 2 controls, 2 references, 6 treatments) in three replicates were calculated. The effects of treatments, storage time and their interactions on colour attributes, myoglobin levels and TBARS values were compared by analysis of variance (ANOVA) using the MIXED model procedure of the SAS 9.4 program (SAS Institute, USA). Treatment, time and their interactions were set as fixed factors and the replication was set as a random factor. Tukey test procedure was used to do multiple comparisons between individual treatments. Correlation analysis among various variables was conducted using CORR procedure. The level of significance was set at $P < 0.05$. The effects of independent variables and their interactions on dependent variables are shown in section 9.3.

5.4 Results and Discussion

The composition of major chemical components of all the burgers samples were described in section 4.4.1. Briefly, the moisture content of burgers ranged from 60.3 to 66.5% while protein content ranged from 14.6 to 16.4%, the fat content of ranged from 16.3 to 18.5% (target fat content of 17%), and the ash content ranged from 1.6 to 1.7%. The pH of these products ranged from 5.5 to 5.6, with an average pH value of 5.54.

5.4.1 Meat colour

During the 12 weeks of frozen storage, all burger treatments suffered gradual discoloration on the surface (shown in Figure 5.1). Among all samples, the controls had the fastest decrease in a^* value, and there was no significant difference ($P>0.05$) of a^* value between control 1 and control 2 burgers. Burgers with added lentil flour had generally higher ($P<0.01$) a^* values than the control burgers throughout the storage. Especially at the end of storage (week 12), burgers with 6% flour from raw or heat-treated lentil maintained their a^* values higher than 20, a reference line for the redness of fresh beef, while the control burgers had lost their redness (below 20) since week 6. This capability to maintain red colour of beef burger may be attributed to the high amount of phenolic compounds in lentil flour compared with other legume seeds (Han & Baik, 2008), and the high antioxidant activity as described in the Study 1. From our observation, lentil flour contains water soluble phenolics at the level of 1.98 - 4.21 mg GAE/g DW sample (see section 3.4.5). Phenolic compounds from other plant sources, such as rosemary extracts added into beef burgers have proven effective in protecting meat colour during frozen storage (-18 °C) for up to 6 months (Georgantelis et al., 2007). In this particular study, a^* value of the control beef burger samples decreased from approx. 18 to 10 in the first 90 days of the frozen storage while samples containing rosemary extracts retained higher a^* value, above 15.

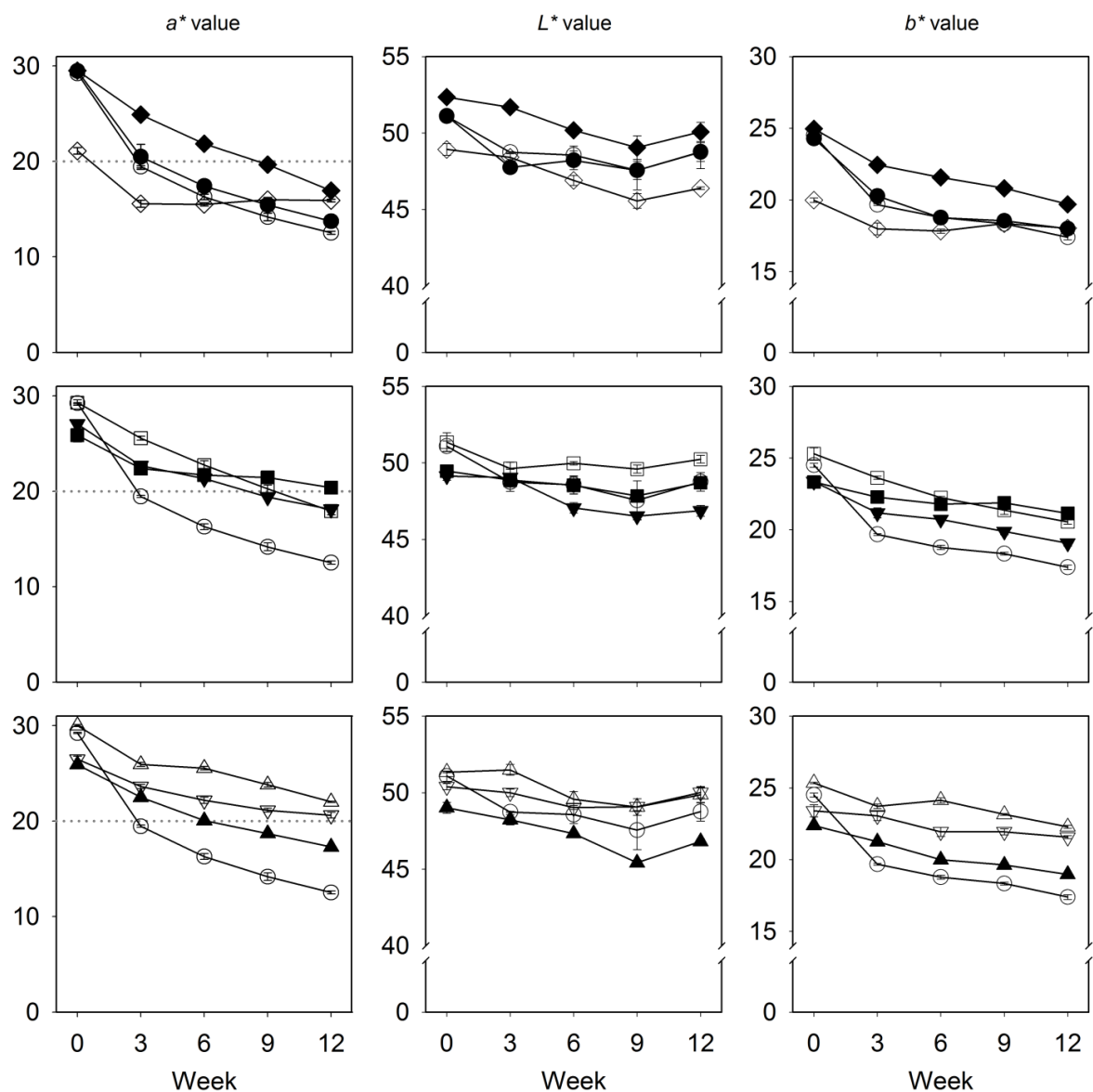


Figure 5.1 Colour changes (a*, L* and b* values) of all beef burger samples stored at -20 °C for 12 weeks. ○ Control 1, ● Control 2, ◇ Sodium erythorbate-0.05%, ◆ Toasted wheat crumb-6.0%, □ Raw lentil, cotyledon-5.4%, ■ Raw lentil, whole seed-6.0%, △ Heat-treated lentil, cotyledon-5.4%, ▲ Heat-treated lentil, seed coat-0.6%, ▽ Heat-treated lentil, whole seed-6.0%, ▼ Raw lentil, seed coat-0.6%

Throughout the storage, the burgers containing heat-treated lentil flour showed no significant ($P>0.05$) difference in the a^* value compared to the burgers with raw lentil flour. In contrast, use of raw lentil flour containing a high activity of oxidative enzymes, such as lipoxygenase and peroxidase, lead to faster discoloration of beef burgers than the controls when stored at 4 °C, as shown in the previous sections of this thesis (see section 3.4.2.1 and 4.4.2). Both lipoxygenases and peroxidases may contribute to faster and elevated lipid oxidation (Burnette, 1977; Eriksson, 1982). The products of lipid oxidation such as 4-HNE may contribute to the oxidation of oxymyoglobin and cause discoloration of meat products (Faustman et al., 2010). However, such discoloration effect was not observed in the frozen burger samples prepared with flour from raw lentil. Most likely it may be due to the inhibited activity of lipoxygenase and peroxidase (Azcarate & Barringer, 2010) at frozen temperature (-20 °C) and also less free water to satisfy reaction conditions on the meat particle surface of the burgers.

A whole lentil seed can be separated into cotyledon and seed coat and each of them accounts for approximately 90% and 10% of the seed weight, respectively. It was found that burgers with the cotyledon flour (5.4%) showed higher ($P<0.001$) a^* values than burgers with whole seed (6.0%) and seed coat (0.6%) flour on week 0. It was noticeable that the addition of seed coat transferred its dark brown colour to the beef burgers. As fine flour particles, seed coat flours had redness values (a^* value) of 6.6 (raw) and 9.6 (heat-treated) and the cotyledon flour were 16.4 (raw) and 7.7 (heat-treated). Working with rosemary extracts, Georgantelis and group (2007) have reported a similar colour transferring effect from the plant extract to beef burgers at the beginning of frozen storage. In their study, the initial a^* value of burger with 0.02% (w/w) rosemary extract was lower than that of the control burger. In our study, generally burgers with added seed cotyledon flour showed higher ($P<0.001$) a^* value than burgers with added seed coat flour. The burgers with added cotyledon flour also showed higher ($P<0.05$) a^* value than the control in the week 12. For burgers with raw cotyledon flour, it could be attributed to the activity inhibition of oxidative enzymes at sub-zero temperatures and the presence of phenolic compounds. For burgers

with heat-treated cotyledon flour, it can be due to the extended antioxidant ability of the phenolic compounds of heat-treated flours. An improvement of ferrous ion chelating ability after heat treatment from 0.0% to 33.9% was reported in the section 3.4.4.5 of Study 1. The burgers with added heat-treated cotyledon flour actually exhibited even higher ($P<0.05$) a^* value than those with raw cotyledon flour on week 12, which could be related to the combination of loss of oxidative enzyme activity and ability to chelate catalytic metal ions of the water soluble components of these flours.

Throughout the frozen storage, addition 0.05% sodium erythorbate caused very low redness values and the burger surface was dark purple. Such low redness is interesting because the burgers with added sodium erythorbate was regarded as a positive control for this study. Under refrigerated storage, ascorbic acid (sodium erythorbate is the sodium salt of erythorbic acid, a stereoisomer of ascorbic acid) has been able to protect meat colour successfully throughout storage (Sánchez-Escalante et al., 2001) and it was further confirmed by the data of Study 2. The burgers with 0.05% sodium erythorbate retained their red colour with a^* value above 20 for 7 days at 4 °C storage (see section 4.4.2). However, adding sodium erythorbate caused a different effect for burger colour under under frozen condition. Suman et al. (2005) also found such performance difference between refrigerated and frozen storage when 0.04% of sodium erythorbate was added into ground beef. Under refrigerated storage, the sodium erythorbate can protect meat colour and maintained a higher a^* value than in an all meat control (see section 4.4.2) while under frozen storage, it caused a purple colour resulting in a lower a^* value (16.6) compared with the control (25.8). However, it was noticed that upon complete thawing, the bright red colour of beef burgers with 0.05% sodium erythorbate was restored. For burgers with 6.0% TWC, the a^* value was higher ($P<0.05$) than the control starting at week 3, suggesting its ability to retain red colour of the beef burger surface under frozen storage condition. This is different from the results under the refrigerated storage condition showing that adding 6.0% TWC did not slow down discoloration compared with the all meat control burger (see section 4.4.2).

For the lightness (L^* value), no difference ($P>0.05$) was found between experimental samples (with added lentil flours) and control burgers throughout the storage period. Also, there was no significant difference ($P>0.05$) between burgers with added heat-treated lentil flour and those with added raw lentil flour throughout the 12 week storage. In contrast, the burgers with added cotyledon components showed higher ($P<0.05$) L^* value (49.9 - 50.2) than those with added seed coat (46.8 - 46.9). It is probably because the colour difference between cotyledon flour and seed coat flour that were incorporated into the beef burgers. Generally, seed coat flour of red lentil always has a very low lightness (approx. 50) displaying dark brown colour (Xu, Yuan, & Chang, 2007). From our observation, the L^* value of seed coat flour of red lentil ranges from 48.6 - 51.2. In comparison, the cotyledon flour used in this work displayed a bright yellow/orange colour with L^* value at 86.5 - 86.9. A positive b^* value represents the yellowness of the surface of beef burgers (AMSA, 2012). The yellowness (b^* value) of beef burgers with added heat-treated lentil flour are higher than burger samples with added raw lentil flour ($P<0.01$). The burger incorporated with cotyledon showed higher b^* value than the burgers with added seed coat ($P<0.001$). Therefore, the colour of different lentil components might have an effect on the surface colour of beef burgers.

5.4.2 Myoglobin redox states

The myoglobin redox states of all samples during the 12-week storage is shown in Figure 5.2. In general, there was a decline in oxymyoglobin level in the surface of all the samples and as a result, an increase in metmyoglobin occurred during frozen storage.

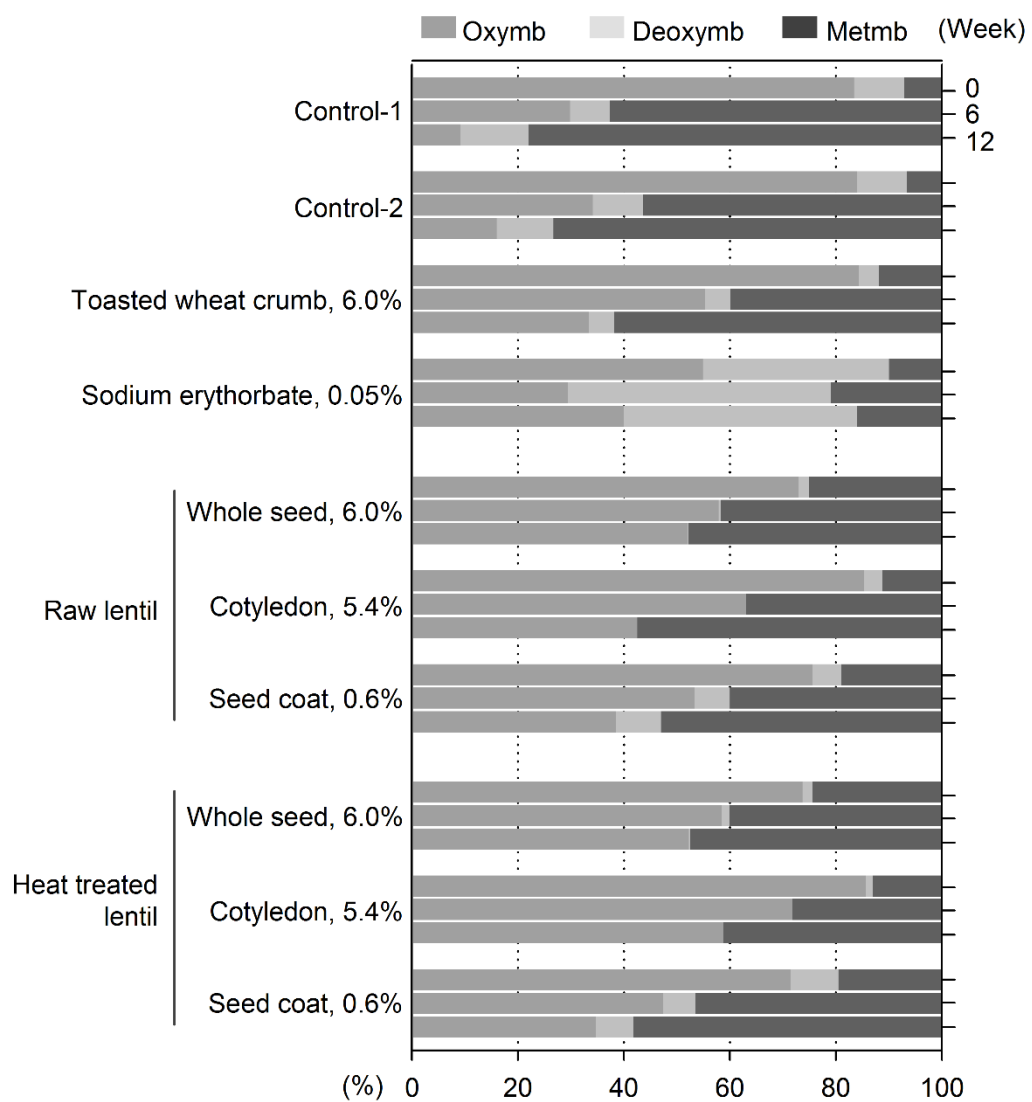


Figure 5.2 Myoglobin redox states of beef burger samples during frozen storage of 12 weeks at -20 °C.

For most samples, except burgers with added sodium erythorbate, deoxymyoglobin was very low (0.0 - 12.9%) and did not change much during storage. The increase in metmyoglobin is due to the denaturation of globin moiety of the myoglobin molecule during freezing and frozen storage, leading to an increased susceptibility of myoglobin to autoxidation (Chen, 2003). Also, the metmyoglobin reducing activity can be lost during frozen storage and subsequent thawing, causing an accumulation of metmyoglobin (Yamanaka, Takamizawa, & Amano, 1973). The two control samples showed very fast oxidation of oxymyoglobin during the 12 week storage without any significant difference ($P>0.05$) between them. The oxymyoglobin level dropped from 83 - 84% at the start of frozen storage to 9 - 10% by the end of storage with an accumulation of metmyoglobin at 73 - 78% on week 12. However, all the experimental samples (burgers with added flours of whole seed, cotyledon and seed coat, with or without heat treatment) retained higher ($P<0.05$) oxymyoglobin level and lower ($P<0.05$) metmyoglobin level than the controls at the same time. Such antioxidant ability could come from the high level of water soluble phenolic compounds (1.98 - 4.26 mg gallic acid/ g DW sample) present in lentil flour as shown in our previous study (see section 3.4.5). It was found that phenolic compounds, such as catechin, chlorogenic acid, rosmarinic acid, kaempferol, etc. when added individually at the levels of 300 and 600 $\mu\text{mol/L}$ in a model system can reduce metmyoglobin into oxymyoglobin (Inai et al., 2014). Lentil is rich in catechin, epicatechin, kaempferol and other phenolic compounds (mainly in the forms of flavonoid glycosides) (Zhang et al., 2015), and make it a highly possible reducing source for metmyoglobin in the beef burger environment during frozen storage. In the model system work (Study 1), water extracts of lentil seed coat which contained high level of phenolic compounds had the capability to reduce metmyoglobin to form oxymyoglobin and caused a 20% decrease in the metmyoglobin level (see section 3.4.6.1). As a result, it is highly possible that phenolic compounds present in lentil can act as reducing agents to reduce metmyoglobin while frozen storage.

Metmyoglobin level of the burgers with added heat-treated lentil whole seed flour did not differ significantly ($P > 0.05$) from that of the burgers with added raw lentil flour. This is probably because the activity of oxidative enzymes was inhibited at $-20\text{ }^{\circ}\text{C}$ (Damodaran, Parkin, & Fennema, 2007), lipid oxidation caused by these oxidative enzymes was depressed, leading to less oxidative stress and less metmyoglobin formation. Under refrigerated conditions, on the other hand, adding raw lentil flour caused a faster formation of metmyoglobin in beef burgers in our previous studies (see section 4.4.2). This is possibly due to the fast lipid oxidation which can produce radicals and oxidation products such as 4-HNE that will enhance the oxidation of oxymyoglobin.

Among burger samples with added heat-treated lentil flour, those with 5.4% added cotyledon flour generally showed significantly lower ($P < 0.01$) metmyoglobin level than those with 0.6% added seed coat flour. Such results explained the higher redness value of burgers with added heat-treated cotyledon flour compared with the burgers with added heat-treated seed coat flour as shown in Figure 5.1. It has been shown that lentil seed coat contained a higher level of total phenolic content than that of cotyledon (25.8 - 38.2 mg/g DW GAE versus 0.4 - 4.3 mg/g DW GAE) (see section 3.4.5). The antioxidant activity measured by DPPH radical scavenging assay showed that the antioxidant activity of lentil seed coat is 500 times higher than that of cotyledon (Boudjou et al., 2013). We also observed the soluble proteins extracted from lentil seed coat showed over 90 times higher activity in DPPH radical scavenging assay than that of cotyledon (see section 3.4.4.2). Higher phenolic compounds may promote the antioxidant activity and contribute to the protection of myoglobin oxidation (Miura et al., 2014), as a result, a lower level of metmyoglobin formation could be expected. However, the addition of lentil seed coat flour with such higher level of phenolic compounds did not result in a lower level of metmyoglobin level of beef burgers. It could be explained by the fact that the amount of seed cotyledon (5.4%) added into burgers was nine times of the amount of seed coat (0.6%) added, so the total antioxidant capacity contributed by cotyledon might be equivalent to or even higher than that of

seed coat. Also, some of the phenolic compounds that are present in seed coat rather in cotyledon, including caffeic acid, kaempferol and quercetin can adversely react with oxymyoglobin and cause it to be oxidized (Masuda et al., 2013).

Interestingly, the burger with 0.05% sodium erythorbate showed a very high deoxymyoglobin level (>35%) starting from the beginning and throughout the whole storage. Such high amount of deoxymyoglobin affected its colour and made the burger surface a dark purple colour. Suman et al. (2005) also found this phenomenon caused by adding 0.04% sodium erythorbate into beef burgers under frozen storage. It is reported that applying ascorbic acid can slow down the oxidation of oxymyoglobin of the beef steak at 4 °C (Mitsumoto, Cassens, Schaefer, & Scheller, 1991). A model system also demonstrated the strong reducing ability of ascorbic acid on metmyoglobin leading to the formation of oxymyoglobin as a result (Tsukahara & Yamamoto, 1983). However, in this case, a de-oxygenation process happened to oxymyoglobin and thus deoxymyoglobin formed causing a dark purple colour of the burger surface. Such de-oxygenation process from oxymyoglobin to deoxymyoglobin was believed to take place under anaerobic conditions, such as vacuum packaging (Mancini & Hunt, 2005). It is not known exactly why such high deoxymyoglobin formed during frozen storage for burgers with 0.05% sodium erythorbate. Maybe the presence of sodium erythorbate resulted in a loss of the oxygen molecule in the oxymyoglobin structure, leading to the formation of deoxymyoglobin and a dark purple colour of the surface of beef burger. It is also worthy to note that after thawed, the purple burgers retained their bright red colour, suggesting an oxygenation process occurred from deoxymyoglobin to oxymyoglobin. For burgers with 6.0% toasted wheat crumb, the metmyoglobin level was no lower ($P>0.05$) than that of the control 2 throughout the frozen storage. The a^* value of burgers with TWC also showed a fast loss during this period (see section 5.4.1).

In the present study, colour and myoglobin measurements on frozen beef burgers were conducted within 5 min, yet unpredictable oxidation of oxymyoglobin could still happen during the measuring operation. For instance, warming up (thawing) in order to take colour

measurements and then freezing of the samples every three weeks may accelerate myoglobin oxidation and discoloration of the burger surface. This process may have impacted the results of instrumental colour measurement and myoglobin redox states resulting in greater changes in colour than would be found under commercial conditions for the same storage period.

5.4.3 TBARS

The level of lipid oxidation of beef burgers during frozen storage was tested using the TBARS assay by determining the secondary products of lipid oxidation, mainly malondialdehyde (St. Angelo et al., 1996). The changes of TBARS value of all treatments during 12-week storage are shown in Figure 5.3. A general increase in TBARS values during the frozen storage took place in most of the samples. The two controls were not different from one another ($P>0.05$) throughout the storage. On week 12, control burgers had TBARS values of 2.35 - 3.05 mg MDA/kg sample while TBARS values of all the samples with added binders were below 2 mg MDA/kg sample. In a study conducted by Georgantelis et al. (2007), TBARS values of the control beef patties (containing 15% fat) also increased rapidly during 90 day storage at -18 °C, from less than 0.5 mg MDA/kg sample to around 3 mg MDA/kg sample.

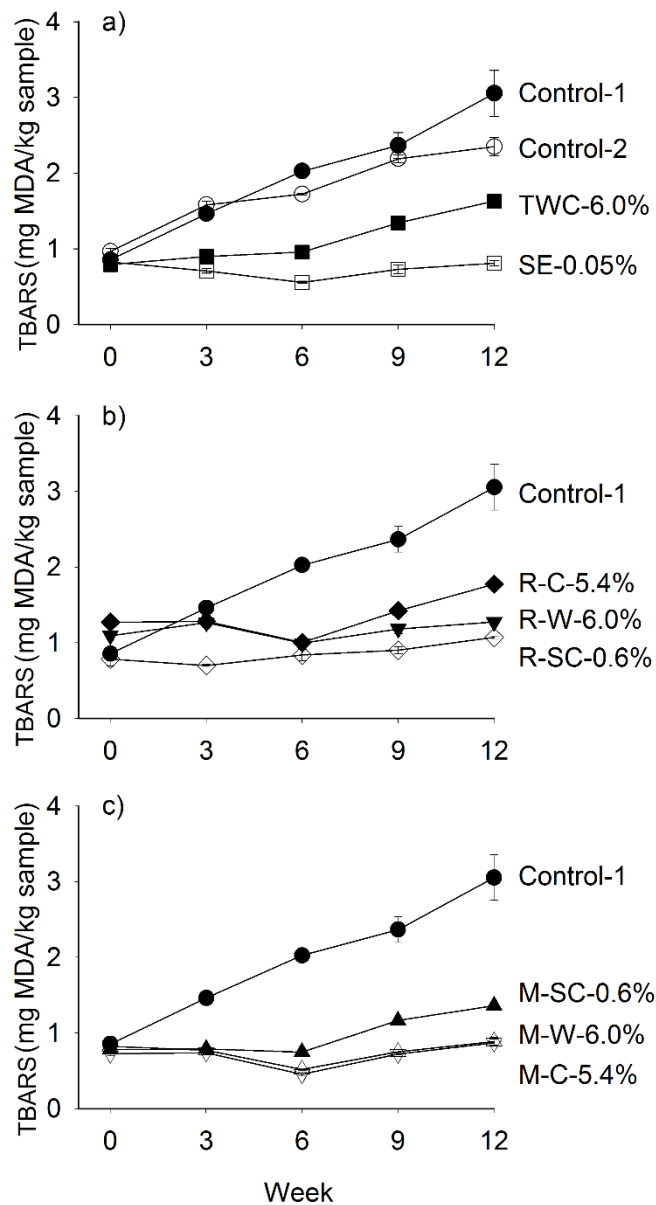


Figure 5.3 TBARS of all beef burger samples stored at -20 °C for 12 weeks, a) controls and reference samples, TWC - Toasted wheat crumb, SE - Sodium erythorbate; b) Samples with raw lentil seeds, R - Raw lentil, C - Cotyledon, W - Whole seed, SC - Seed coat; c) Samples with heat-treated lentil seeds, M - Heat-treated lentil.

The burgers with incorporation of lentil components all showed lower ($P < 0.05$) TBARS values than the control 1 starting at week 6, indicating their ability to retard lipid oxidation during frozen storage. The antioxidant activity of lentil mainly comes from phenolic compounds (Oomah et al., 2011). According to Study 1, the water soluble phenolic compounds in whole lentil seed flour is 1.98 - 4.21 mg GAE/g DW sample (see section 3.4.5). Researchers have found that phenolic compounds in lentil seed are the major antioxidants contributing high ability to scavenge radicals and to chelate metal ions (Oomah et al., 2011) that could slow down lipid oxidation within the beef burgers under frozen conditions. Some researchers also investigated the effect of adding herbal extracts which contain phenolic compounds into meat products during frozen storage. Rojas and Brewer (2008) studied the extracts from grape seed, rosemary oleoresin and oregano and their effect on beef patties at level of 0.01 - 0.02% during 4 month frozen storage at -18 °C. The results indicated that 0.02% grape seed extract and 0.02% oregano extract can slow down lipid oxidation compared with the control. In another study, Georgantelis et al. (2007) added rosemary extract at 200 mg/kg, chitosan at 10 g/kg and tocopherol at 60 mg/kg into beef burgers and tested their effect on colour and lipid oxidation during 5 month frozen storage at -18 °C. The antioxidant extracts successfully lowered the lipid oxidation from 5 mg MDA/kg (control) to around 2 mg MDA/kg. The antioxidant abilities of phenolic compounds rely on several functions: ability of metal binding, inhibition ability of reactive oxygen species producing enzymes and inhibition effect on lipid autoxidation (Dangles, 2012). In the present study, TBARS values remained under 2 mg MDA/kg sample, showing strong antioxidant results, comparable to burgers with 0.05% sodium erythorbate. When comparing with the use of herbal extracts as antioxidants, lentil flours bring more advantages because of the multiple functions they provide. As a meat binder, lentil flour contains high level of protein, starch and fibre that improved texture and cook yield (Der, 2010). It also contains phenolic compounds (free and bound to protein) that can prevent lipid oxidation and protect meat colour as shown in Studies 1, 2 and 3.

There is no difference ($P>0.05$) in TBARS values among all experimental burger samples from week 0 to week 9. It is noteworthy that on week 12, burgers with added raw cotyledon flour showed higher ($P<0.05$) TBARS value than burgers with added heated cotyledon flour. This suggests that even though the frozen condition of $-20\text{ }^{\circ}\text{C}$ inhibited the activity of oxidative enzymes in flour from raw lentil seed with respect to oxymyoglobin oxidation and metmyoglobin formation, they still had an adverse effect on meat product quality to some extent. Such adverse effect was also found in the Study 2 in which raw lentil flour exhibited higher TBARS values than heat-treated flour to beef burgers stored at $4\text{ }^{\circ}\text{C}$ (see section 4.4.4). Bahçeci et al. (2005) reported that after months of frozen storage, the lipoxygenase and peroxidase in green beans can still be active upon thawing. This may be the reason that higher TBARS value found from the burgers with raw lentil cotyledon, because the results of Study 1 (see section 3.4.2.1) showed that cotyledon contains much higher lipoxygenase activity than seed coat. As a result, oxidative enzymes are still a potential threat to the quality of meat particles of the burgers in contact with raw lentil flour (whole seed or cotyledon), especially upon thawing, the effect would be greater than under frozen conditions. From the result of a preliminary test, it was observed that beef burgers with added raw lentil flour exhibited faster discoloration than control and the burgers with heat-treated lentil flour in display case when previously frozen burgers were thawed and displayed under refrigerated condition after 2-week frozen storage.

Under frozen conditions, raw lentil flour did not affect colour changes or oxymyoglobin oxidation of beef burgers, but had an unfavorable effect on lipid oxidation compared with heat-treated lentil flour. Under refrigerated conditions, however, incorporating raw lentil flour had negative effects on both oxymyoglobin oxidation and lipid oxidation (see section 4.4.3 and 4.4.4). These results indicated that the oxidative enzymes present in raw lentils have a direct effect on accelerating lipid oxidation, and by producing more lipid oxidation products, accelerate oxymyoglobin oxidation in an indirect way. Once the activity of these enzymes is suppressed

under frozen condition, the acceleration impact on discoloration of beef burgers may not be fully expressed.

The burgers with added lentil cotyledon flour (both raw and heat-treated) do not have significantly different TBARS values ($P>0.05$) from those with added seed coat flour. This is probably because the amount of cotyledon component added into beef burgers was 5.4% while the seed coat component was added at 0.6% only. Even though the level of total phenolic compounds in seed coat was 9 - 14 times higher than that of cotyledon, they contribute similar antioxidant effect to prevent lipid oxidation when considering their proportional amount (cotyledon, 90% and seed coat, 10%) in lentil whole seed (as shown in section 3.4.5). Such results also suggest that lentil seed coat is a natural source of phenolic compounds that can separately be used as an ingredient in meat product formulations at relatively low level (eg. 0.6%) in order to provide antioxidant activity.

The burgers with 0.05% sodium erythorbate (SE) showed significantly lower ($P<0.05$) TBARS values than the control burgers starting at week 6. The TBARS values of these burgers were below 1.0 mg MDA/kg sample throughout the storage, suggesting a strong antioxidant activity of sodium erythorbate. Burger samples with heat-treated lentil flour (whole seed, 6.0%) showed similar TBARS values as the burger sample with 0.05% SE, indicating their similar antioxidant activity on inhibiting lipid oxidation in beef burgers. The burgers with 6.0% toasted wheat crumb (TWC) also showed lower ($P<0.05$) TBARS values than control burgers starting at week 6. This is possibly because there are some phenolic compounds (approx. 0.7 - 0.9 mg ferrulic acid eq./g defatted sample) present in wheat flour (Liyana-Pathirana, & Shahidi, 2006).

Twelve weeks of frozen storage is a short storage duration compared with some of studies conducted by previous researchers. Brewer and Harbers (1991), Bhattacharya, Hanna and Mandigo (1988), or Georgantelis et al. (2007) have used storage time periods of 39 weeks, 20 weeks and 26 weeks, respectively. In the present study, surface colour, metmyoglobin level and

TBARS values between the controls and the experimental samples became very distinctive starting at week 6. Hence, such period of storage is sufficient to show the results very clearly. However, it would be interesting to see the duration of the antioxidant effect with longer storage.

5.4.4 Correlation analysis

The correlation analysis among variables is shown in Table 5.1. The a^* value is highly correlated with b^* value ($r = 0.93$), while negatively correlated with metmyoglobin level ($r = -0.86$) and TBARS values ($r = -0.61$). The weaker correlation between a^* value and TBARS values could result from the slower progression of TBARS values compared with the rapid changes of surface colour during the frozen storage of 12 weeks. Also, the instrumental colour measurement was conducted on the surface of burger samples while the TBARS values were determined on the whole piece of a burger. The metmyoglobin level is correlated with TBARS value while is negatively correlated with oxymyoglobin and deoxymyoglobin levels. Correlations between a^* and b^* ($r = 0.70$), a^* and metmyoglobin level ($r = -0.74$) were also found in frozen stored ($-18\text{ }^{\circ}\text{C}$ for 52 weeks) lean ground beef by Brewer and Wu (1993). They also found negative correlations between a^* value and metmyoglobin. A negative correlation between a^* value and TBARS value ($r = -0.863$) was also observed by Georgantelis et al. (2007) in frozen stored beef burgers ($-18\text{ }^{\circ}\text{C}$ for 180 days).

Table 5.1 Correlation coefficients among dependent variables measured on beef burgers during frozen storage

	a^*	L^*	b^*	MetMb	OxyMb	DeoxyMb
L^*	0.57***					
b^*	0.93***	0.63***				
MetMb	-0.86***	-0.46***	-0.74***			
OxyMb	0.59***	0.18*	0.46***	-0.61***		
DeoxyMb	0.66***	0.42***	0.65***	-0.80***	0.28***	
TBARS value	-0.61***	-0.16*	-0.52***	0.60***	-0.49***	-0.44***

Note: *refers to significant correlation ($P < 0.05$), *** refers to significant correlation ($P < 0.001$)

5.5 Conclusions

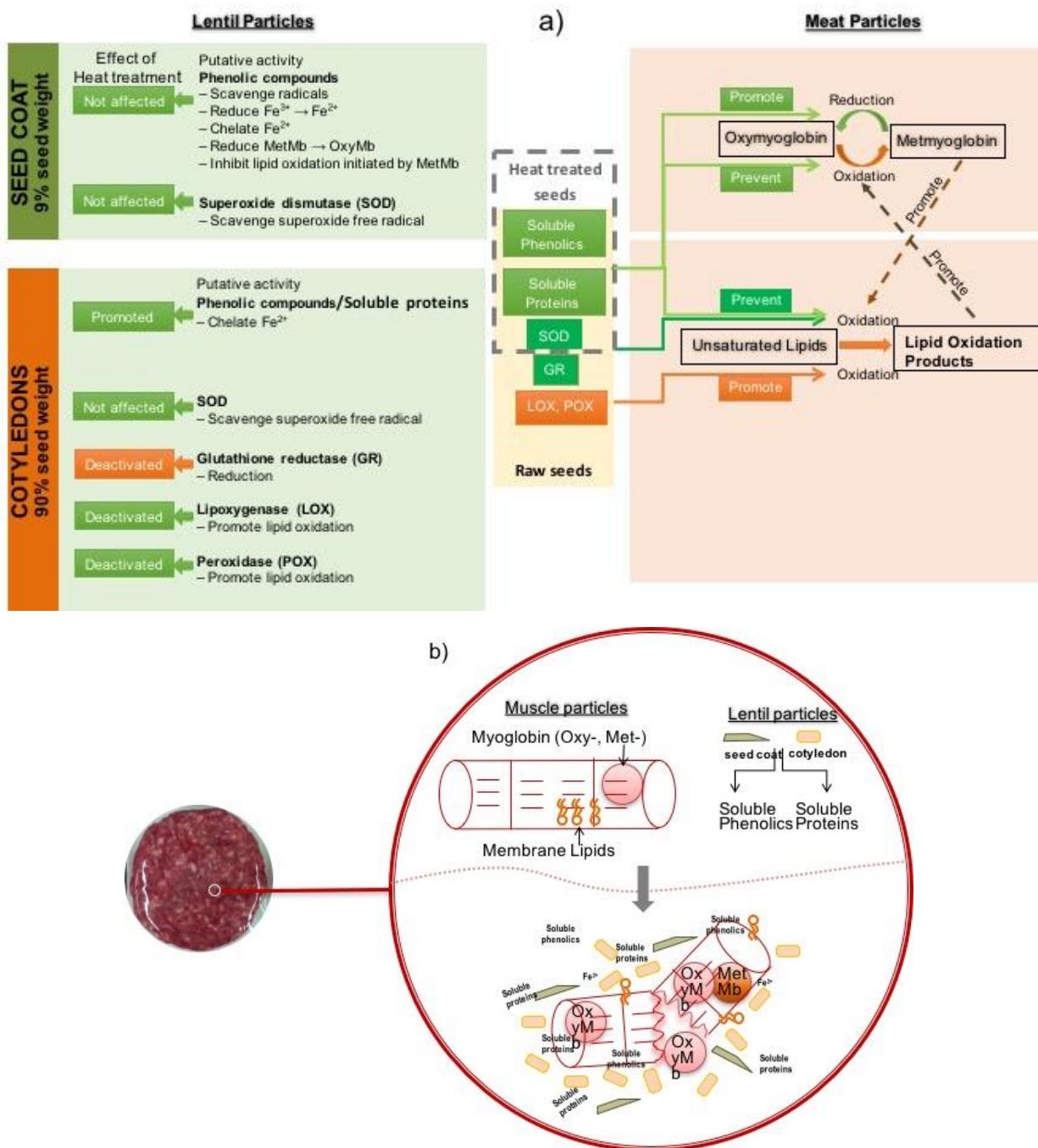
During frozen storage, the addition of lentil flour (whether or not heat treated) slowed down the decrease in surface redness (a^* value > 20) and the development of metmyoglobin, and retarded lipid oxidation (lower TBARS values) compared to control burgers composed of meat only during the frozen storage. Adding flour of heat-treated or raw lentils did not cause a difference on a^* value of beef burgers during the storage. However, burgers with added seed cotyledon from heat-treated lentil seeds showed lower metmyoglobin level and higher redness than those with seed coat which is an advantage. Adding heat-treated lentil flour led to lower TBARS values of burgers than those with added raw ones, likely due to the lower activity of oxidative enzymes such as lipoxygenase in the heat-treated lentil flours. No difference ($P>0.05$) was found on the TBARS values between the burgers contained flours of cotyledon (5.4%) and seed coat (0.6%), probably due to their similar contribution of water soluble phenolics. Adding sodium erythorbate caused a purple colour of beef burgers during frozen storage but lowered TBARS values. Toasted wheat crumb also showed some antioxidant ability to protect colour and to retard lipid oxidation.

The results indicated that incorporating heat-treated lentil flour can prolong the redness of frozen beef burgers and can retard lipid oxidation during frozen storage. Although adding raw lentil flour can protect colour during frozen storage, it did not inhibit lipid oxidation of beef burgers to the same extent as heat-treated lentil flour did. Together with its textural benefits to beef burgers, more bland flavour and low activity of oxidative enzymes observed from studies 1 and 2, heat-treated whole lentil flour is a multi-functional meat binder that can extend storage life of frozen beef burgers. Considering the huge market size of frozen ground beef products, it will be beneficial to utilize heat-treated lentil flour to improve the qualities of ground beef products during frozen storage.

6 GENERAL DISCUSSION

In summary, this study investigated antioxidative and oxidative activities of the soluble components of whole lentil seed and seed physical components and the effect of heat treatments on these activities, in order to investigate the impact of adding lentil flour to raw beef burger formulations in terms of surface colour parameters and lipid stability. Beef burgers were stored in both refrigerated and frozen conditions.

Lentil seed mainly contains two distinct, physically separable parts which are cotyledon and seed coat (Scheme 1a). Cotyledon accounts for approximately 90% of the seed weight and contains a high amount of protein and starch as well as fibre. Seed coat accounts for approximately 9 - 10% of the seed weight and contains a lower amount of protein, and soluble carbohydrates, but is high in fibre content. The distribution of oxidative and antioxidative enzyme activities between these two components are different. The activities of lipoxygenases, peroxidases and glutathione reductase are greater in the cotyledons while superoxide dismutase activity is greater in the seed coat (see section 0 and 3.4.2.1) thus contributing to the differences in total antioxidant potential of each fraction when evaluated separately.



Scheme 1 a) Effect of heat treatment (left) on phenolic compounds, antioxidative and oxidative enzymes within seed coat and cotyledon separately and their effects on the oxidation of oxymyoglobin and lipid in meat system (right). **b)** Proposed burger system (below) with broken muscle cell and released myoglobin species and lipids, and with the presence of incorporated lentil particles (seed coat and cotyledon) containing soluble proteins and phenolics

The phenolic compounds, the major antioxidative molecules of lentil seeds are not evenly distributed between the seed coat and cotyledons. The level of phenolic compounds, soluble in water or ethanolic solvents, are much higher in the seed coat than in the cotyledon (see section 3.4.5). Seed coat is a more concentrated source of antioxidant molecules and has a higher potential to provide antioxidant activity than cotyledons as pointed out using various model assays including radical scavenging ability (DPPH and ABTS), ferric reducing ability, ferrous ion chelating ability, metmyoglobin reducing ability and metmyoglobin initiated linoleic acid oxidation activity.

Other contributors to the antioxidant capacity of lentil may include water soluble peptides/proteins, tocopherols and other minor components (Fernandez-Orozco et al., 2003). According to Zhang et al. (2014), the total tocopherol (α -, γ - and δ - tocopherol) content of red lentil (Maxim) whole seed is 47.7 $\mu\text{g/g}$ DW seed and green lentil (Greenland) is 64.4 $\mu\text{g/g}$ DW seed. Although the level of tocopherol was not measured for the lentil samples of the present study, the total phenolic content determined ranged from 1.40 mg/g DW (70% ethanol, v/v) and 4.21 mg/g DW (water) for red lentil.

Beef is the meat source for this study because it is a commonly consumed meat containing a much higher level of myoglobin than other types of meat such as pork and chicken (Lawrie & Ledward, 2006). Such higher level of myoglobin makes beef more sensitive to the impact of pro-oxidants and antioxidants present in the meat system and in the minced or ground form the myoglobin conversions are accelerated. The model meat product used in the current study is the beef burger, which was prepared mainly using ground beef (fat or lean) which has large surface area exposed to oxygen. This meat model also represents similar particle size of meat and ingredients to that of fresh non-cured sausages. Other ingredients used in this model product include water, salt and binder/antioxidants. However, all other unnecessary ingredients such as spices and flavorants were not included to exclude unwanted reactions. The addition of salt is not only because it is a common ingredient, but also it performs as an oxidation accelerator for both

lipids and myoglobin (Andersen & Skibsted, 1991) and helps in bringing some of the dilute salt solution soluble components into the reaction sphere. The flat patty shape of beef burgers makes it suitable for colour measurement. The use of ground beef also makes the beef burger system vulnerable to oxidative factors, such as radiation due to illumination, free radicals and free metal ions and access to oxygen.

It is not well-documented how exogenous enzymes affect the colour and lipid oxidation of raw meat or raw meat products. An early study describing negative effects of legumes on meat colour by Verma et al. (1984) reported that adding chickpea flour into sheep meat sausage increased the formation of metmyoglobin and elevated TBARS values. They attributed this phenomenon to the presence of lipoxygenase present in chickpea. Applying heat treatment to the legume eliminated such negative effects by deactivating the enzyme activity. Lentil has been reported to possess higher activity of lipoxygenase than chickpea (Chang & McCurdy, 1985), so it can also contribute in enhancing oxidation reactions in meat products when raw lentil flour is used as a binder.

In this study, the oxidative enzymes from raw lentil can cause meat discoloration in beef burgers stored at refrigeration temperature (see section 4.4.2 and 4.4.3). This can be seen from the faster decrease in a^* value of burgers with added raw lentil flour (whole seed and cotyledon) compared with the burgers with added heat-treated lentil flour. Even the burgers with added 6% whole lentil flour from raw/heat-treated (50:50, w/w) showed a similar faster decrease in a^* value to that of burgers with all raw whole lentil flour. This result suggests that a complete deactivation of lipoxygenase and peroxidase is necessary prior to using lentil flour as a meat binder. The main substrates of lipoxygenases are the unsaturated lipids in the product rather than myoglobin. It seems that lipoxygenase did not directly affect the oxidation rate of oxymyoglobin in the meat system, but the effect is by interfering with lipid oxidation. The interrelations between lipid oxidation and myoglobin oxidation are well documented (Faustman et al., 2010). The products of lipid oxidation such as 4-HNE can accelerate the oxidation of oxymyoglobin while the products

of oxymyoglobin oxidation including metmyoglobin may induce the oxidation of lipids (Faustman et al., 2010).

Superoxide dismutase, on the contrary, contributes to the total antioxidant capacity of lentil flour. In a model system, Gorelik and Kanner (2001b) found that superoxide dismutase can decrease the oxidation rate of oxymyoglobin, but has no effect on lipid oxidation. It is difficult to specifically determine the contribution of superoxide dismutase as its activity is not measured by standard antioxidant assays, only the scavenging of superoxide radicals is measured. Its ability to scavenge other radicals or to chelate metal ions could be very low compared with some of the potent antioxidants. As it scavenges superoxide radicals though, it can protect the cell or the whole matrix from being oxidized.

It is also interesting to notice the opposing performances of raw lentil flour on beef burgers under refrigerated and frozen storage conditions (see section 4.4 and 5.4). Raw lentil flour (whole seed flour and cotyledon flour) has adverse effects on colour and lipid oxidation when beef burgers were stored under refrigerated conditions. However, under frozen conditions, the colour was not affected during the 12 week of storage, but higher TBARS was observed at the end of storage for the burger samples incorporated with raw lentil (cotyledon flour or whole seed flour) compared with the burgers containing heat-treated lentil flour. It can be speculated that the refrigerated condition did not stop the activity of enzymes. So those oxidative enzymes still work and caused an increase in lipid oxidation of the raw meat products under refrigeration. The products of lipid oxidation as a result accelerated myoglobin oxidation and produce much darker and browner surface colour. On the other hand, under the frozen condition, it seems that the enzymes within the raw lentil flour were still slightly active towards their substrates and caused an increase in the rate of lipid oxidation (compared with heat-treated samples, but still slower than the control groups). However, the relationship between the lipid oxidation products and myoglobin oxidation was likely broken, because of the presence of ice crystals which could block

the migration of lipid oxidation products to the site of myoglobin. As a result, the colour change is not as negative as observed in the refrigerated condition.

Phenolic compounds from lentil seeds can protect colour of meat products in several ways (see section 3.4.4 and 3.4.6). During the storage of meat products either under refrigerated or frozen condition, oxymyoglobin will be gradually oxidized with the presence of oxygen by oxidizing agents present in the meat system. As a result, the amount of metmyoglobin keeps going up and the colour of raw meat products will turn brown. However, with the addition of phenolic compounds, part of the metmyoglobin can be reduced to form oxymyoglobin at the same time as metmyoglobin is being continually formed. Such effect of phenolic compounds should be the main source of power of seed coat to protect meat colour from discoloration in raw meat products. Meanwhile, phenolic compounds can scavenge radicals and chelate metal ions and limit the oxidative stress in the system. In this way, lipid oxidation can be lowered and less lipid oxidation products would promote the oxidation of oxymyoglobin. A third mechanism is also described in the literature, but not evaluated in the present study. Zhang, Li, Meng, He, & Ren (2016) showed that phenolic compounds (from mulberry leaf extracts, at 1 mg/g meat) can elevate the activity of SOD and glutathione peroxidase in raw beef patties stored at 4 °C for 13 days.

The model system developed used metmyoglobin as the substrate to evaluate the reducing ability of lentil extracts (see section 3.4.6.1). The results showed that lentil seed coat extracts can reduce metmyoglobin to form oxymyoglobin. In fact, oxymyoglobin is the major form of myoglobin when the raw meat product is freshly made. So is it possible that phenolic compounds prevented the oxidation of oxymyoglobin? Not likely. According to the results of Masuda et al. (2013), phenolic compounds added into the model system containing oxymyoglobin actually caused a decrease in the oxymyoglobin level and formation of metmyoglobin. This is possibly because phenolic compounds could be oxidized to quinones and these quinones can oxidize

oxymyoglobin and form metmyoglobin. So the major colour protection effect of phenolic compounds must come from their reducing ability on metmyoglobin.

It is known that seed coat contains higher amount of phenolic compounds and as a result, contributes to higher antioxidant power than cotyledons. From the results of actual meat products, we observed a relatively low a^* value at the beginning for the burgers with incorporated seed coat (see section 4.4.2). Such lower a^* value is not preferable because this phenomenon not only affected the colour comparison between treatments, but also affected the application of seed coat in meat products. It is assumed that such lower a^* value comes from the colour migration from seed coat itself. This is because the seed coat is a green/brown colour and when it is added to raw beef, it may bring the green/brown colour into the final product and alters the colour. Some researchers found such effects as well. The extracts of chamnamul (*Pimpinella brachycarpa* (Kom.) Nakai) and fatsia (*Aralia elata* Seem) contains antioxidants but are greenish colour which affects the beef colour (Kim et al., 2013). Curry and mint leaf extracts affect the colour of pork patties on day 0 because they displayed greenish colour (Biswas et al., 2012). The elm-leaf blackberry extracts showed a red colour and almost made the redness value of the pork patties double (Ganhão et al., 2010). Therefore, a similar colour migration effect may have interfered with the colour of meat products at the initial stage of storage and should be avoided using a tangible strategy.

Phenolic compounds are effective antioxidants on retarding lipid oxidation either in meat system or in linoleic acid oxidation model system. The addition of lentil seed coat flour can suppress the development of TBARS values of the beef burgers under 1.5 mg MDA/kg sample during the refrigerated and frozen storage, while the control burgers showed TBARS values over 3 mg MDA/kg sample during both types of storage. Raw meat products are complex systems containing high level of lipids. Among all lipids, unsaturated fatty acids are easy to be oxidized through self peroxidation or under the effect of internal or external enzymes, metal ions and radicals. Phenolic compounds are potent antioxidants that can scavenge radicals, reduce ferric ion

to ferrous ion and chelate metal ions as shown in our study. By doing these actions, phenolic compounds might decrease the oxidation stress of the whole system, no matter in the meat system such as beef burgers or in a model system composed of liposome and oxymyoglobin.

Another interesting finding is the counteracting effect of phenolic compounds and oxidative enzymes when comparing the a^* values, metmyoglobin levels and TBARS values among burgers with added raw lentil flour of whole seed, cotyledon and seed coat during the refrigerated storage (see section 4.4). Obviously, burgers with added seed coat showed higher a^* value, lower metmyoglobin level and lower TBARS than those with cotyledon. Moreover, as a combination, burgers with added whole seed just showed results exactly in middle point of those containing seed coat or cotyledons. The flour from the whole seed contained the equivalent seed coat components and cotyledon components but the results did not track close to that observed for burgers with either component alone. This intermediate effect suggests a simple counteracting effect of antioxidants and oxidative enzymes on the results of colour and lipid oxidation. When oxidative enzymes in raw lentil accelerated oxidation of lipids and oxymyoglobin of raw meat, the presence of phenolic compounds acted as a decelerator to slow down the oxidation pace of the meat pigment and unsaturated fatty acids. In addition, phenolic compounds can also react with these oxidative enzymes to inhibit their activities (Laughton et al., 1991; Baynton et al., 1994) and protect meat colour and inhibit lipid oxidation as a result.

Heat treatment such as infrared heating to 150 °C mainly deactivated lipoxygenase, peroxidase and glutathione reductase, but did not change superoxide dismutase activity (Scheme 1a). This is an important finding because lentil seed contains a combination of oxidative and antioxidative enzymes. By applying heating, no matter whether it is direct (infrared heating) or indirect (water bath heating), these oxidative enzymes can be deactivated. The remaining superoxide dismutase mainly contributes to antioxidative power extending to meat products. The heat treatment also caused a sharp increase in ferrous ion chelating ability of cotyledon from 0 to

over 25%. As a result, thermal treatments brings down oxidative potential of lentil seed which is a great advantage when lentil flour is added to meat products as a binder.

Data for the colour attributes of beef burgers incorporated with heat-treated flours (whole seed, cotyledon and seed coat) stored in refrigerated condition indicated that these flours all showed various degrees of colour protection ability compared with the control burger (as shown in section 4.4.2). It is reasonable that the flour of whole seed and seed coat showed the colour protection effect on beef burgers, but the flour of cotyledon also showed a similar ability. One possible explanation is that the level of addition of cotyledon (5.4%) and seed coat (0.6%), so more or less equal total phenolic compounds according to their TPC of water soluble extracts are provided. Under refrigerated storage, on the day 7 of storage, the TBARS value of burgers with added cotyledon flour (3.21 mg MDA/kg sample) is twice than that of burgers with added whole seed (1.25 mg MDA/kg sample) and seed coat (1.21 mg MDA/kg sample) suggesting that the water soluble phenolic compounds may not be the only contributor to the observed colour protection effect. Another possible explanation for the colour protection of heat-treated lentil cotyledon could come from the metal binding capacity of soluble protein. In section 3.4.4.5, it is found that soluble proteins extracted from lentil cotyledons showed a dramatic increase ($P < 0.05$) in ferrous ion chelating ability upon heat treatment, while the radical scavenging ability did not change. This is possibly because of the structure change of soluble proteins under heat treatment and more chelating sites are exposed as a result. It is believed that type II antioxidants (metal chelators) are more likely to protect oxymyoglobin from being oxidized than type I antioxidants (radical scavengers) (Allen & Cornforth, 2009). So this may be the reason why heat-treated cotyledon can protect meat colour, but cannot inhibit lipid oxidation to the same degree as flours from whole seed or seed coat.

Unlike oxidative enzymes, extractability of phenolic compounds of whole seeds were not highly affected by heat treatment, such as infrared heating that was used in the study (see section 3.4.5). However, decrease in solubility of these compounds differs with the seed physical

components and the solvent used. For the cotyledons, basically no difference was found in the level of TPC before and after heat treatment in either ethanolic solution or water as solvent. For the seed coat, on the other hand, after heat treatment the TPC level in 70% (v/v) ethanol decreased but no difference is found for water extract. It is believed that thermal processing can cause degradation of phenolic compounds, due to enhanced oxidation and breaking of covalent bonds (Nayak et al., 2015). For example, anthocyanins degrade to form smaller molecules, such as aldehydes and benzoic acid derivatives. This degradation of original phenolic compounds caused a decrease in antioxidant activity at the beginning which was then followed by an increase at later stages. This suggested that new antioxidants formed and the antioxidant activity was elevated as a result. For this study, such change of antioxidants may occur to the phenolic compounds in lentil as well.

All seeds prior to heat treatment were tempered to 23% moisture in order to assist heating process and to prevent burning or charring of seeds during heat treatment. During infrared heat treatment, more moisture loss could occur than the seeds in vacuum packaged bags in water bath heating. As a result, more Maillard reaction could occur during infrared heating than in water bath heating. In different types of heat treatments, no difference was found in antioxidant activities of soluble proteins extracted from different samples, suggesting the Maillard reaction products may not be the major source of antioxidant activity.

With this evidence, lentil flour can be considered a good source of binder/filler for applications in refrigerated meat products. It is recommended that heat-treated whole seed flour could be used in refrigerated products prepared with raw, ground meat such as burgers, patties and meat balls. This is because the heat-treated lentil flour can improve cooking yield and water holding capacity, and provide a bland flavour and antioxidant capability in protecting colour and preventing lipid oxidation. All these features make heat-treated whole lentil flour a multifunctional binder for minced meat products. Meanwhile, the use of raw lentil flour, especially raw cotyledon flour of lentil should be avoided in the applications of refrigerated

minced meat products. This is because not only the beany flavour will be brought into the meat products, but an added risk of colour deterioration and elevated level of lipid oxidation may occur.

In the frozen meat products such as beef burgers that will be sold as frozen, there is no difference in colour stability between raw or heat-treated lentil flour that can be highlighted from the present study. One consideration is there could be more lipid oxidation (higher TBARS value) which was found for the burgers with added raw lentil flour than burgers with added heat-treated lentil flour during frozen storage. Therefore, the use of heat-treated lentil flour in frozen meat products is also recommended.

If textural modification and product extension are not the aim, then seed coat might be the choice as an antioxidant because of its high antioxidant power that can prolong colour and can prevent lipid oxidation in either raw meat products, under refrigeration or frozen conditions. In this study, the level of seed coat added into beef burgers was at 0.6% and it performed well in colour protection and inhibition of lipid oxidation. Also, unlike the other commercial sources of phenolic compounds, such as green tea extracts, grape seed extracts or rosemary extracts which are obtained by extracting with organic solvents, lentil seed coat is a natural source of phenolic compounds without the need for extraction, and may not contribute to inherent strong flavours as noted with some of the commercial extracts.

This study provides necessary evidence for the fundamental understanding of the effect of lentil flour on meat colour protection and lipid oxidation prevention. It provides the insight on the complexity of lentil seed components, differences in how they were modified due to heat treatment and consequently having different effects on oxidation reactions of myoglobin and lipids in raw meat products. A better understanding of the complexity of lentil flour components and their relations with meat components would be beneficial for future applications. In this work, different levels of phenolic compounds were found in seed coat and cotyledon. The

molecular species found under phenolic compounds could also be different in seed coat and cotyledon that will affect the colour protection and inhibition of lipid oxidation in meat products. Therefore, a survey on the different types of phenolic compounds from seed coat and cotyledon in different solvents and characterizing their molecular components would be beneficial.

Moreover, it could be fruitful to explore the direct effects of exogenous enzymes, such as lipoxygenase and superoxide dismutase on meat colour changes, myoglobin oxidation and lipid oxidation. In this work, lipoxygenase and peroxidase were mainly located in the cotyledon of raw lentil seeds and they are considered responsible for the faster discoloration of raw beef burgers and higher lipid oxidation level found during refrigerated and frozen storage. In a preliminary test, adding pure lipoxygenase and peroxidase into raw beef burgers caused a similar faster discoloration compared with the control. Since the substrate of lipoxygenase is mainly poly unsaturated fatty acids, the oxidation of lipids should have a direct link with oxymyoglobin oxidation that caused the discoloration. It is believed that the product of lipid oxidation, such as 4-HNE can accelerate the oxidation of oxymyoglobin, resulting in discoloration of meat products (O'Grady et al., 2001). Hence, it would be desirable to investigate the direct effect of lipoxygenase on the colour changes of beef burgers or on oxymyoglobin oxidation with or without the presence of unsaturated fatty acids.

In addition, more study could be conducted regarding the use of lentil seed coat in meat products. In this work, 0.6% lentil seed coat flour was added into beef burgers and colour protection and inhibition of lipids were found in both refrigerated and frozen condition. But as we know, the colour protection is concentration dependable. It is not known what the optimum level of seed coat would be for specific applications. Consequently, it would be very helpful to evaluate the effect of adding different levels of lentil seed coat flour as a source of natural antioxidants/phenolic compounds as well as a pool of fibre in the application in meat products to protect colour and to inhibit lipid oxidation.

7 GENERAL CONCLUSIONS

This study investigated the antioxidative effect of lentil flour on the protection of raw meat colour and inhibition of lipid oxidation under refrigerated and frozen storage conditions and tried to find out the reasons for different effects of various lentil flours on the colour changes and lipid oxidation of meat products.

Lentil seed is a complex entity, physically composed of an outer layer consisting of seed coat and an inner part that of cotyledon. It is also a combination of oxidative enzymes and components possessing various antioxidant activities. It is found that seed coat contributes more antioxidant power than the cotyledon when tested at the same level, mainly coming from phenolic compounds, protein-bound phenolics and superoxide dismutase. However, since cotyledon accounts for 9 - 10 times the weight/mass of seed coat within lentil seed, the water soluble phenolic content are comparable between these two components on a whole seed basis. Meanwhile, cotyledon contains high activity of oxidative enzymes, namely lipoxygenase and peroxidase, contributing dramatic acceleration effect on lipid oxidation when added into raw meat products.

Heat treatments such as infrared heat treatment as a pre-treatment of lentil seeds can eliminate the oxidative enzymes. Also, heat treatment has little effect on either antioxidative enzymes or phenolic compounds which are the major antioxidants in lentil. Furthermore, heat treatment caused a sharp increase in ferrous ion chelating ability of soluble proteins of cotyledon, likely contributing colour protection ability as a type II antioxidant (metal chelator).

The incorporation of lentil flour in beef burgers stored in refrigerated condition demonstrated the antioxidant ability of lentil flour. Beef burgers containing raw cotyledon flour which contains

high level of oxidative enzymes caused a faster discoloration and higher TBARS values compared with those with added heat-treated flour or seed coat flour. The colour changes in burgers containing raw cotyledon is the result of oxymyoglobin oxidation and formation of metmyoglobin, due to the fast development of lipid oxidation induced by these oxidative enzymes. While under frozen condition, these enzymes only slightly increased lipid oxidation but has no effect on myoglobin oxidation or colour changes. In fact, all the burger samples containing lentil flour showed higher a^* value and lower TBARS value than the control samples at the end of 12 week frozen storage. The antioxidant effect of the phenolic compounds and soluble proteins in meat colour protection and lipid oxidation prevention involve the ability to chelate free metal ions, to scavenge peroxy radicals, to reduce metmyoglobin to from oxymyoglobin, and to prevent linoleic acid oxidation initiated by metmyoglobin.

Therefore, the application of heat-treated whole lentil flour in raw meat products can protect the colour and prevent lipid oxidation either in refrigerated condition or in frozen condition. Together with the characteristics of minor beany flavour, increasing cooking yield and improved water holding capacity, the heat-treated whole lentil flour provides significant antioxidant ability and is a potential binder for meat products.

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9 APPENDIX

9.1 Supplemental data for Chapter 3

Table 9.1 Probability levels of main factors and their interactions for lentil seed composition

Independent variables	Dependent variables			
	Moisture	Ash	Protein	Fat
Cultivar	0.17	<0.01	<0.01	0.06
Heat treatment (HT)	<0.05	<0.01	0.86	0.77
Seed component	<0.01	<0.01	<0.01	<0.01
Cultivar × HT	<0.01	0.54	0.82	0.34
Cultivar × Seed component	0.47	<0.05	<0.05	0.81
HT × Seed component	<0.05	<0.01	<0.05	0.32
Cultivar × HT × Seed component	0.55	0.47	0.97	0.85

Table 9.2 Probability levels of main factors and their interactions for enzyme activities of lentil

Independent variables	Dependent variables			
	SOD	GR	LOX	POX
Cultivar	0.70	<0.05	<0.01	<0.01
Heat treatment (HT)	0.06	<0.01	<0.01	<0.01
Seed component	<0.01	<0.01	<0.01	<0.01
Cultivar × HT	0.77	<0.01	<0.01	<0.01
Cultivar × Seed component	0.83	0.08	<0.01	0.09
HT × Seed component	<0.01	<0.01	<0.01	<0.01
Cultivar × HT × Seed component	0.89	<0.05	<0.01	<0.05

Table 9.3 Probability levels of main factors and their interactions for antioxidant activities of water soluble extracts

Independent variables	Dependent variables			
	DPPH	ABTS	FRAP	Fe ²⁺ chelating
Cultivar	0.06	<0.05	<0.01	0.37
Heat treatment (HT)	0.10	<0.05	0.38	<0.01
Seed component	<0.01	<0.01	<0.01	<0.01
Cultivar × HT	0.76	0.51	0.68	0.33
Cultivar × Seed component	0.12	0.17	<0.01	0.16
HT × Seed component	<0.01	<0.01	<0.01	<0.01
Cultivar × HT × Seed component	0.91	0.818	0.69	0.39

9.2 Supplemental data for Chapter 4

Table 9.4 Probability levels of main factors for compositional analysis of all samples stored in refrigerated condition

Independent variables	Dependent variables				
	Moisture	Ash	Protein	Fat	pH
Sample	<0.001	<0.05	<0.001	<0.01	<0.001

Table 9.5 Probability levels of main factors and their interactions for colour of burger samples added with lentil flour stored in refrigerated condition

Independent variables	Dependent variables		
	a*	b*	L*
Heat treatment on lentil seeds (HT)	<0.01	0.2352	0.1948
Seed component	0.0636	<0.001	<0.001
Storage time (Day)	<0.001	<0.001	<0.001
HT × Seed component	0.1775	0.221	<0.001
HT × Day	<0.001	<0.001	<0.001
Seed component × Day	<0.001	<0.05	<0.001
HT × Seed component × Day	<0.001	<0.01	<0.001

Table 9.6 Probability levels of main factors and their interactions for myoglobin redox states and TBARS values of burger samples added with lentil flour stored in refrigerated condition

Independent variables	Dependent variables			
	OxyMb	MetMb	DeoxyMb	TBARS
Heat treatment on lentil seeds (HT)	<0.01	<0.001	<0.001	<0.05
Seed component	<0.05	<0.01	<0.001	<0.001
Storage time (Day)	<0.001	<0.001	<0.001	<0.001
HT × Seed component	0.1249	0.4023	<0.001	0.0707
HT × Day	<0.001	<0.001	<0.001	0.0733
Seed component × Day	<0.001	<0.01	<0.001	<0.001
HT × Seed component × Day	<0.001	<0.05	<0.001	0.2996

Table 9.7 Beef burger treatments and formulation for supplemental samples

Treatments and binder levels	Meat /g	Binder /g	Ice water /g	Salt /g	Total /g
Raw lentil, whole seed, 6.0%	85.0	6.0	8.1	0.9	100.0
Raw:Heat-treated (1:1), whole seed, 6.0%	85.0	6.0	8.1	0.9	100.0
Heat-treated lentil, whole seed, 6.0%	85.0	6.0	8.1	0.9	100.0
Heat-treated lentil, whole seed, 0.6%	85.0	0.6	13.5	0.9	100.0
Heat-treated lentil, cotyledon, 0.6%	85.0	0.6	13.5	0.9	100.0
Heat-treated lentil, seed coat, 0.6%	85.0	0.6	13.5	0.9	100.0

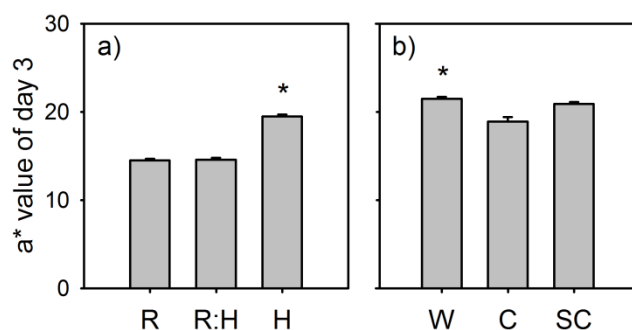


Figure 9.1 a) a* value of burgers with added 6.0% whole seed (r: raw lentil, h: heat-treated lentil, r:h: raw:heated = 1:1) on day 3; b) a* value of burgers with added 0.6% heated treated lentil components (W: whole seed, C: cotyledon, SC: seed coat) on day 3.

Note: * indicates significant difference ($P < 0.05$) from other samples

9.3 Supplemental data for Chapter 5

Table 9.8 Probability levels of main factors and their interactions for colour of burger samples added with lentil flour stored in frozen condition

Independent variables	Dependent variables		
	a*	b*	L*
Treatment (Raw or heat treatment)	0.1193	<0.05	0.1268
Seed component	<0.001	<0.001	<0.001
Storage time (Day)	<0.001	<0.001	<0.01
Treatment × Seed component	<0.05	<0.001	0.4171
Treatment × Day	0.5051	0.1812	<0.05
Seed component × Day	<0.01	<0.001	<0.05
Treatment × Seed component × Day	<0.05	<0.01	<0.01

Table 9.9 Probability levels of main factors and their interactions for myoglobin redox states and TBARS values of burger samples added with lentil flour stored in frozen condition

Independent variables	Dependent variables			
	OxyMb	MetMb	DeoxyMb	TBARS
Treatment (Raw or heat treatment)	<0.05	0.3284	0.9482	<0.001
Seed component	<0.001	<0.001	<0.001	0.2391
Storage time (Day)	<0.001	<0.001	<0.001	<0.001
Treatment × Seed component	<0.001	<0.05	0.2863	<0.001
Treatment × Day	0.1655	0.5081	0.8398	0.8653
Seed component × Day	<0.001	<0.05	<0.001	<0.01
Treatment × Seed component × Day	<0.01	<0.05	0.2466	<0.05